Increased Mutagen Sensitivity and DNA Damage in Pulmonary Arterial Hypertension

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Abstract

Rationale: Pulmonary arterial hypertension (PAH) is a serious lung condition characterized by vascular remodeling in the precapillary pulmonary arterioles. We and others have demonstrated chromosomal abnormalities and increased DNA damage in PAH lung vascular cells, but their timing and role in disease pathogenesis is unknown.

Objectives: We hypothesized that if DNA damage predates PAH, it might be an intrinsic cell property that is present outside the diseased lung.

Methods: We measured DNA damage, mutagen sensitivity, and reactive oxygen species (ROS) in lung and blood cells from patients with Group 1 PAH, their relatives, and unrelated control subjects.

Measurements and Main Results: Baseline DNA damage was significantly elevated in PAH, both in pulmonary artery endothelial cells ($P < 0.05$) and peripheral blood mononuclear cells (PBMC) ($P < 0.001$). Remarkably, PBMC from unaffected relatives showed

similar increases, indicating this is not related to PAH treatments. ROS levels were also higher ($P < 0.01$). DNA damage correlated with ROS production and was suppressed by antioxidants ($P < 0.001$). PBMC from patients and relatives also showed markedly increased sensitivity to two chemotherapeutic drugs, bleomycin and etoposide ($P < 0.001$). Results were consistent across idiopathic, heritable, and associated PAH groups.

Conclusions: Levels of baseline and mutagen-induced DNA damage are intrinsically higher in PAH cells. Similar results in PBMC from unaffected relatives suggest this may be a genetically determined trait that predates disease onset and may act as a risk factor contributing to lung vascular remodeling following endothelial cell injury. Further studies are required to fully characterize mutagen sensitivity, which could have important implications for clinical management.

Keywords: DNA damage; reactive oxygen species; genetic susceptibility; BMPR2

Pulmonary arterial hypertension (PAH) is defined as a resting mean pulmonary artery pressure of greater than 25 mm Hg with normal wedge pressure (<15 mm Hg) and a pulmonary vascular resistance

greater than 3 Wood units (1). This results from progressive vascular remodeling in the precapillary pulmonary arterioles and vasoconstriction, and can lead to right ventricular failure. Although treatments

are improving, it remains a life-threatening condition (2, 3). PAH may be heritable (HPAH), idiopathic (IPAH), or associated (APAH) with an underlying condition, such as congenital heart

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At a Glance Commentary

Scientific Knowledge on the

Subject: There is mounting evidence of increased DNA damage in pulmonary arterial hypertension (PAH) lung cells, but its timing and relevance to disease pathogenesis is unclear.

What This Study Adds to the

Field: Here we show that peripheral blood cells from patients with idiopathic, heritable, or associated PAH also show elevated levels of DNA damage and are significantly more sensitive to damage induced by chemotherapeutic drugs. Similar results were found in cells from patients' unaffected relatives, suggesting this may be a novel genetically determined phenotype that predates the development of PAH.

defect or connective tissue disease (4). HPAH is caused by a heterozygous mutation in one of several genes, most often bone morphogenetic protein type-II receptor (*BMPR2*) (5–7). It is inherited as an autosomal-dominant trait, but shows reduced penetrance, meaning that only about 27% of individuals who carry a mutation actually develop PAH in their lifetime (8). This suggests that other genetic and/or environmental factors are necessary to trigger the disease. The molecular basis of other types of PAH remains largely unknown.

The first evidence for somatic genetic changes in PAH lungs was the identification that endothelial cells within plexiform lesions, a pathognomonic feature of PAH, may be monoclonal and show evidence of microsatellite instability (9–11). Subsequently, we identified large-scale cytogenetic abnormalities in a small series of pulmonary artery endothelial cells (PAEC) isolated from the explant lungs of patients with PAH (12). These abnormalities were also detectable in the uncultured lung tissue by fluorescent *in situ* hybridization (12, 13), confirming they are not culture artifacts and suggesting there may be a higher rate of DNA damage and/or mitotic segregation errors in PAH lungs. Increased DNA damage has been identified in PAH lung

vascular cells (14–16). However, it remains unknown whether this phenomenon is an early event that may contribute to vascular remodeling, or is a secondary bystander resulting from the abnormal lung milieu of advanced PAH. The goal of this study was to determine if PAH cells are intrinsically more susceptible to DNA damage and whether this damage is confined to the diseased lung, or is a more widespread phenomenon that might predate the disease. Some of the results of these studies have been previously reported in the form of abstracts (17, 18).

Methods

Study Population

PAEC, pulmonary microvascular endothelial cells (MVEC), and pulmonary artery smooth muscle cells (PASMC) were isolated from the explant lungs of patients with PAH as previously described (19) and maintained in EGM-2 or SmGM-2 media (Lonza, Allendale, NJ), respectively. Control cells were isolated from failed donor lungs or purchased commercially (Lonza). Demographic and clinical details are included in the online supplement in the METHODS section and Table E1. Analysis for mutations in *BMPR2*, *SMAD9*, and *CAV1* genes was performed by sequencing and multiplex ligation-dependent probe amplification, as previously described (20, 21), and for *ACVRL1*, *ENG*, *KCNK3*, and *EIF2AK4* using a custom Haloplex kit (Agilent, Santa Clara, CA). Chromosome abnormalities were identified using single-nucleotide polymorphism (SNP) arrays (cytoSNP-12 v2; Illumina, San Diego, CA), as previously described (12).

Lymphoblastoid cell lines (LCL) were established from patients, first-degree relatives, and unrelated control subjects by Epstein-Barr virus (EBV) transformation of peripheral B lymphocytes and maintained in RPMI-1640 with 20% fetal bovine serum. Demographics of the study population are shown in Table E2. Fresh blood samples were later obtained from seven of these subjects, together with a large independent group of patients, first-degree relatives, and control subjects for validation studies (see Table E3). All subjects provided written informed consent under protocols approved by the Cleveland Clinic Institutional Review Board.

DNA Damage Assays

Micronucleus (MN) assay was conducted in PAEC, LCL, or fresh peripheral blood mononuclear cells (PBMC), with or without antioxidants, and scored by light microscopy. Detailed methods are included in the online supplement. Results are the average of at least two replicate experiments. Validation studies were performed on fresh PBMC by flow cytometry (22), counting the number of MN per 50,000 intact nuclei (see supplementary METHODS). Parallel aliquots of cells treated with etoposide, bleomycin, or griseofulvin were compared with untreated cells. Phosphorylation of histone H2AX at serine-139 (γ H2AX) was analyzed in PAEC and LCL by immunofluorescence and confocal microscopy (see supplementary METHODS). The index of double strand breaks (DSB) was calculated as the ratio between area of γ H2AX staining and total nuclear area, averaged across 70–100 cells per case.

Reactive Oxygen Species

Reactive oxygen species (ROS) production was measured in PAEC and LCL by flow cytometry using CellROX Green (Life Technologies, Grand Island, NY), as detailed in the online supplement. Mitochondrial superoxide production in PAEC was measured by MitoSOX Red (Life Technologies) and hydrogen peroxide production by Red Hydrogen Peroxide kit (Enzo Life Sciences, Farmingdale, NY) in microplates. Triplicate measurements were averaged for each case. Results were normalized to the number of live cells. Further details are provided in the supplementary METHODS.

Statistics

Statistical analyses were performed using Prism v5 (GraphPad Software, La Jolla, CA). Two-sample tests were performed using Student *t* test or Mann-Whitney rank sum after testing for normal distribution and equal variance. Likewise, multiple group analyses were performed with one-way analysis of variance (ANOVA) or ANOVA on ranks (Kruskal-Wallis), followed by *post hoc* pairwise tests. The effects of antioxidants and sensitivity to genotoxic drugs were analyzed by two-way ANOVA with repeated measures. Where there was a significant interaction, *post hoc* tests were performed for each treatment versus untreated cells within each subject group. All graphs are plotted as mean $\pm$ SD and *P* values are two tailed.

Results

Frequency of Chromosome Abnormalities in PAH Lung Vascular Cells

We first profiled the spectrum of chromosome aberrations in a larger series of lung vascular cells. Molecular karyotyping was performed using SNP arrays in PAEC, MVEC, and PASMC from explant PAH lungs and control donor lungs deemed unsuitable for transplant. Copy number changes were identified in 13 of 43 (30.2%) PAH PAEC, compared with only 1 of 19 (5.3%) control cells (Fisher exact test; $P < 0.05$) (Table 1). This series includes the 10 cases and 8 control subjects we published previously (12, 13). In PASMC the frequency of abnormalities was not significantly different between patients (5 of 28; 17.9%) and control cells (one of eight; 12.5%). MVEC were also abnormal in one of three PAH cases, but no control MVEC were available. Five cases had more than one chromosomal change and one had a total of four different changes, three in PAEC and one in PASMC

(Table 1). Of 27 total abnormalities, 12 (44.4%) were rearrangements involving DNA DSB and the remainder were whole chromosome gains or losses. DSB rearrangements were only observed in PAH cells (Table 1). For 11 cases, DNA from other primary lung cells, whole lung tissue, or PBMC was analyzed as a comparison and no abnormalities were detected, confirming that changes in PAEC or PASMC are lineage-specific somatic events (Table 1).

The presence of a chromosome abnormality in pulmonary vascular cells did not correlate with smoking, medication exposures, or PAH severity, as determined by the closest available pretransplant hemodynamics (*see* Tables E4–E6). Although there was no overall difference in age between patient and control cells (*see* Table E1), chromosomally abnormal cells were from significantly older lungs than normal cells ($P < 0.05$) (*see* Table E6). However, this may be skewed by the presence of childhood cases in the chromosomally normal group, because if only adult lungs were considered, the

frequency of chromosome abnormalities in PAH remained significant (13 of 40 PAH vs. 1 of 18 control PAEC; $P < 0.05$) but there was no longer an association with age.

Markers of DNA Damage Are Elevated in PAH PAEC

To investigate whether these abnormalities reflect an ongoing increase in DNA damage, we performed MN assay, a measure of chromosome breakage and loss. An MN is visualized as a small additional nucleus in cytokinesis-blocked binucleate cells (Figure 1A). Four cases were randomly selected from each of three groups: (1) PAH PAEC with a known chromosome abnormality, (2) cytogenetically normal PAH PAEC, and (3) normal control cells. One-way ANOVA was statistically significant ($P = 0.003$) and chromosomally abnormal PAEC had significantly more MN than control PAEC ($P < 0.01$) (Figure 1B). Because the array results suggested a high incidence of DSB, we also analyzed the presence of histone H2AX

Table 1. Chromosomal Abnormalities Detected in Lung Vascular Cells

ID	Status	Sex	Cell Type	Abnormality	% of Cells	Type of Abnormality	Other Tissues Tested Normal
1	Control	F	PAEC	dup(18)	25	Chromosome gain	
2	HPAH	M	PAEC	del(13); del(21)	10–50	Chromosome loss	PASMC, PBMC, BrSMC
3	HPAH	F	PAEC	dup(17)(q22-qter)	75–100	DSB	
4	IPAH	F	PAEC	del(X)	40	Chromosome loss	PASMC, BrSMC
5	IPAH	M	PAEC	del(8)(p23.1-p12)	10	DSB	PASMC, PBMC
6	IPAH	M	PAEC	dup(18)(cen-qter); del(Y)	50	DSB; chromosome loss	PASMC
7	IPAH	M	PAEC	del(Y)	100	Chromosome loss	
8	IPAH	F	PAEC	Chromosome 18 loss or gain	<10	Loss or gain	
9	APAH-CHD	F	PAEC	del(13)(q12.2-q33.3)	75	DSB	PASMC, lung
10	APAH-CHD	F	PAEC	dup(19)	75–100	Chromosome gain	MVEC, lung
11	APAH-CTD	F	PAEC	del(X)	25	Chromosome loss	PBMC
12	PVOD	M	PAEC	del(Y)	50–60	Chromosome loss	
13	IPAH	F	MVEC	dup(12)(pter-p13.31)	50	DSB	PAEC
				dup(19)(pter-cen)	<20	DSB	
14	Control	M	PASMC	dup(18); dup(20)	25–50	Chromosome gain	
15	IPAH	F	PASMC	dup(9)(q21.31-qter)	50	DSB	PAEC
16	APAH-CHD	M	PASMC	del(3)(q28); dup(10)	80–90	DSB; chromosome gain	
17	APAH-CHD	F	PASMC	del(3)(q25.2-q26.1)	60–75	DSB	
18	IPAH	F	PAEC	del(X)	50	Chromosome loss	BrSMC
			PASMC	del(X)	10	Chromosome loss	
19	APAH-CTD	F	PAEC(1)	del(1)(q23.2;q23.3); del(X)	75	DSB; chromosome loss	Lung
			PAEC(2)	dup(11)(cen-qter)	75–100	DSB	
			PASMC	del(8)(q21.11)	30	DSB	

Definition of abbreviations: APAH = associated PAH; BrSMC = bronchial smooth muscle cells; cen = centromere; CHD = congenital heart defect; CTD = connective tissue disorder; del = deletion; DSB = double strand break; dup = duplication; HPAH = heritable PAH; IPAH = idiopathic PAH; MVEC = microvascular endothelial cells; p = short arm of chromosome; PAEC = pulmonary artery endothelial cells; PAH = pulmonary arterial hypertension; PASMC = pulmonary artery smooth muscle cells; PBMC = peripheral blood mononuclear cells; PVOD = pulmonary venoocclusive disease; q = long arm of chromosome; ter = end of chromosome.

Figure 1. DNA damage assays in pulmonary artery endothelial cells (PAEC). (A) Micronucleus (MN) is visualized as additional discrete nuclear material (arrow) in a binucleate (BN) cell in which cytokinesis has been blocked with cytochalasin B. (B) The number of MN per 1,000 BN cells was scored in control PAEC ($n = 4$), pulmonary arterial hypertension (PAH) PAEC with a chromosomal abnormality ($n = 4$), and PAH PAEC that were cytogenetically normal on single-nucleotide polymorphism microarray analysis ($n = 4$). One-way analysis of variance was statistically significant ($P = 0.003$), and chromosomally abnormal PAEC were significantly different than control cells in *post hoc* pairwise testing. $^{**}P < 0.01$. (C and D) Immunofluorescent analysis of histone H2AX phosphorylation (γ H2AX) was performed by confocal microscopy. Results are expressed as the ratio of the area of γ H2AX staining to total nuclear area for at least 70 cells per case. PAH PAEC ($n = 5$) showed significantly more γ H2AX staining than control cells ($n = 3$); t test, $^{*}P < 0.05$. Mean $\pm$ SD. Representative images are shown for control and patient cells. APAH = associated PAH; HPAH = heritable PAH; IPAH = idiopathic PAH.

phosphorylation (γ H2AX), a marker of DSB repair, in PAEC from five patients and three control subjects. Patient cells showed significantly more γ H2AX staining per nuclear area compared with control PAEC ($P = 0.034$) (Figures 1C and 1D). Furthermore, the amount of γ H2AX staining was significantly correlated with MN number in the same cells (Pearson

$r = 0.848$; $P = 0.008$), suggesting that the damage observed by MN assay is primarily caused by DSB (see Figure E1).

Increased Levels of DNA Damage in Peripheral Blood Cells from Patients with PAH and Their Relatives

We then asked whether this increased DNA damage exists outside the lung. First, we

used our bank of EBV-transformed LCL. The control group initially included six unaffected mutation-negative first-degree relatives of patients, along with seven unrelated healthy subjects (see Table E2). Unexpectedly, however, relatives' LCL showed significantly higher MN levels than unrelated control cells (t test; $P < 0.001$). Thus in subsequent analyses,

we considered relatives as a separate group and also included two unaffected mutation carriers. One-way ANOVA was statistically significant ($P < 0.001$), with more MN in LCL from patients and relatives than control subjects ($P < 0.01$) (Figure 2A). Analysis of γ H2AX in eight of the LCL showed a similar trend, but this was not statistically significant (see Figure E2).

To verify that these results are not an artifact of EBV immortalization, we performed two independent validations in fresh PBMC. First by light microscopy, we analyzed 27 patients, 10 relatives, and 18 control subjects; all but seven of these subjects were independent of the LCL group. MN levels in PBMC were significantly higher in patients and relatives than control cells ($P < 0.001$) (see Figure E3A). For the seven individuals represented in both the LCL and PBMC groups, there was a strong correlation between MN counts in the two cell types (Pearson $r = 0.928$; $P = 0.003$) (see Figure E3B). We also performed flow cytometry analysis of PBMC from seven control subjects, nine relatives, and 23 patients (see Table E3), which confirmed significantly more MN in relatives ($P < 0.05$) and patients ($P < 0.001$) than control cells (Figure 2B). There was no difference in the number of MN by sex (t test; $P = 0.74$), nor any correlation with age (Pearson $r = 0.124$; $P = 0.47$). No chromosome abnormalities were detected by SNP array in seven LCL (two control subjects, three PAH, two relatives) or PBMC DNA from four patients.

DNA Damage Correlates with ROS and Is Reversed by Antioxidants

Oxidative stress can lead to mutagenic DNA damage in the form of oxidative base modifications and the induction of DSB. To test if DNA damage in PAH cells is related to oxidative stress, we measured levels of ROS in PAEC and LCL by flow cytometry using CellROX Green, a pan-ROS marker. PAH PAEC showed significantly more ROS-positive cells than control PAEC ($P < 0.001$) (Figure 3A). LCL from patients and relatives also showed significantly more ROS than control cells ($P < 0.01$) (Figure 3B). ROS production correlated with MN levels both in PAEC (Spearman $r = 0.855$; $P < 0.01$) and in LCL (Spearman $r = 0.811$; $P < 0.001$) (see Figure E4), suggesting that increased ROS contributes to elevated levels of DNA damage.

Figure 2. DNA damage assays in peripheral blood cells. (A) The number of micronuclei (MN) per 1,000 binucleate (BN) cells was scored by light microscopy in lymphoblastoid cell lines from control subjects ($n = 7$), patients with pulmonary arterial hypertension ($n = 21$), and unaffected relatives of patients ($n = 8$), including two relatives carrying hereditary pulmonary arterial hypertension mutations. One-way analysis of variance was statistically significant ($P < 0.001$). Patients and relatives had a significantly higher number of MN than control subjects in *post hoc* testing. $**P < 0.01$; $***P < 0.001$. (B) Results were validated by flow cytometry on fresh peripheral blood mononuclear cells from an independent group of control subjects ($n = 7$), relatives ($n = 9$), and patients ($n = 23$). Number of MN was counted per 50,000 intact nuclei. Kruskal-Wallis, $P < 0.001$; *post hoc* pairwise tests, $*P < 0.05$; $***P < 0.001$. Mean $\pm$ SD.

We therefore evaluated whether antioxidants could reduce the level of micronuclei. PBMC were incubated with or without *N*-acetylcysteine (NAC) during the 72-hour MN protocol. This significantly reduced the number of MN in cells from

patients ($n = 21$) and relatives ($n = 10$) to a level similar to that of control cells ($n = 9$) ($P < 0.001$) (Figure 4). Similar results were obtained in LCL treated with NAC or vitamin C (see Figure E5). In contrast, the effect of NAC in PAEC was variable and

Figure 3. Increased reactive oxygen species (ROS) in pulmonary arterial hypertension pulmonary artery endothelial cells and lymphoblastoid cell lines. (A) Pulmonary artery endothelial cells from control subjects ($n = 4$) and patients ($n = 8$) were incubated with CellROX Green reagent and analyzed by flow cytometry for 20,000 events. The percentage of ROS-positive cells was significantly higher in pulmonary arterial hypertension cases than control subjects. Mean $\pm$ SD; $***P < 0.001$. (B) Rate of ROS production in lymphoblastoid cell lines from patients ($n = 10$), relatives ($n = 6$), and control subjects ($n = 7$) was analyzed by flow cytometry. Results are expressed as the change in the percentage of ROS-positive cells among 20,000 counts after 1 hour in antioxidant-free medium compared with baseline, average of at least two independent experiments. Mean $\pm$ SD; one-way analysis of variance, $P = 0.001$; $**P < 0.01$ (*post hoc* pairwise tests).

overall there was no significant reduction in the level of MN (see Figure E6). Notably, however, only one case showed a significant reduction in ROS when treated with 200 μ M NAC (see Figure E6) and thus higher antioxidant concentrations may be required to suppress ROS in PAEC. Mitochondrial superoxide and hydrogen peroxide production were also analyzed in PAEC and were significantly higher in PAH cells than control cells ($P < 0.01$) (see Figure E7).

Increased Mutagen Sensitivity in PBMC from Patients and Their Relatives

We also questioned whether cells from patients with PAH are more sensitive to DNA damaging agents. Three drugs with well-characterized DNA damage profiles were selected to mirror the two types of cytogenetic abnormality we observed in lung vascular cells: etoposide and bleomycin, which induce DNA strand breaks; and griseofulvin, which inhibits spindle microtubules, leading to aneuploidy. PBMC were incubated with or without each drug and then the number of MN was analyzed by flow cytometry. Two-way ANOVA showed a strong interaction between treatment and subject group ($P < 0.001$), with both drugs inducing significantly more MN in PBMC from relatives and patients compared with control subjects (Figure 5). Results were significant across IPAHA, HPAHA, and APAHA subgroups (see Figure E8). In contrast, there was no difference between groups in their sensitivity to griseofulvin (see Figure E9).

Discussion

Several recent studies support an increase in DNA damage in cells from PAH lungs (14–16), but their timing and relevance to disease pathogenesis is unclear. Our results presented here demonstrate that this is not merely a lung-specific effect of end-stage PAH and similar increases in baseline levels of DNA damage are present in peripheral blood cells. Furthermore, PAH PBMC were markedly more sensitive to drug-induced DNA damage. Critically, we show that PBMC from unaffected relatives mirrors the results seen in patients' cells, arguing that this is not an effect of PAH or its treatment and suggesting it may reflect a shared genetic risk factor that predates disease development. Alternatively, it could indicate a strong environmental effect, but this seems less likely given the results

Figure 4. Effect of antioxidant on DNA damage in peripheral blood cells. Fresh peripheral blood mononuclear cells from control subjects ($n = 9$), patients ($n = 21$), and first-degree relatives ($n = 10$) were incubated with 200 μ M *N*-acetylcysteine (NAC) during the 72-hour micronucleus (MN) protocol. The number of MN per 1,000 binucleate (BN) cells was scored by light microscopy and analyzed by two-way analysis of variance with repeated measures. There was a statistically significant interaction between treatment and patient groups ($P < 0.001$). In *post hoc* analyses, NAC significantly reduced the number of MN in cells from patients and relatives but not control subjects; ns = not significant; *** $P < 0.001$.

were consistent across relatives in three generations (parents, siblings, and offspring). In addition, we have partially controlled for shared environment by including spouses of patients in our control group where possible. Metabolic changes

and DNA damage have been associated with *BMP2* mutations (16, 23, 24). However, we found that the incidence of chromosome abnormalities in PAEC and the levels of DNA damage, ROS production, and mutagen sensitivity were

Figure 5. DNA damage induced by genotoxic drugs in peripheral blood mononuclear cells. Induction of micronuclei (MN) by the genotoxic drugs etoposide and bleomycin was measured in peripheral blood mononuclear cells from control subjects ($n = 7$), relatives ($n = 9$), and patients ($n = 23$) by flow cytometry for 50,000 intact nuclei. Untreated cells are the same results as shown in Figure 2B. Mean $\pm$ SD; two-way analysis of variance interaction, $P < 0.001$. *Post hoc* tests were performed for drug versus untreated cells in each group; ns = not significant; ** $P < 0.01$, *** $P < 0.001$.

Figure 6. Model illustrating the potential role of DNA damage in pulmonary arterial hypertension (PAH). Individuals with normal genotype have low levels of reactive oxygen species (ROS) and DNA damage and normal restoration of the pulmonary vasculature after endothelial injury. Individuals with the PAH-susceptible genotype produce higher levels of ROS (red triangles) and have higher baseline levels of DNA damage, both in the lung and in white blood cells (blue circles). This may lead to an increase in the number of pulmonary artery endothelial cells with unrepaired genomic damage (highlighted in gray). On endothelial cell injury and death, cells with preexisting genomic damage may be resistant to apoptosis and primed for hyperproliferation. If the injury itself includes ROS generation or other genotoxic damage, cells with the PAH-susceptible genotype are also more sensitive to the DNA-damaging effects, thus amplifying the extent of the injury compared with normal cells. As vascular remodeling and pulmonary hypertension progresses, hypoxia, abnormalities of mitochondrial metabolism, inflammation, and oxidative stress may lead to additional DNA damage and create a vicious cycle of ongoing endothelial and smooth muscle cell dysfunction.

all consistently elevated across HPAH, IPAH, and APAH, and did not correlate with mutations in *BMPR2* or other HPAH genes. Thus, our results suggest that susceptibility to DNA damage is a widespread phenomenon in PAH, shared among multiple subtypes with seemingly disparate etiologies.

Alterations in oxygen sensing and redox balance are now well recognized in pulmonary hypertension, including abnormal mitochondrial number and morphology, oxidative stress, and a shift to glycolytic metabolism (23–31). However, this study is the first to directly link increased ROS production in PAH cells to elevated levels of DNA damage. We also showed that PBMC from patients and their relatives are significantly more sensitive to the DNA-damaging effects of etoposide and bleomycin, but not griseofulvin. Bleomycin is a radiomimetic drug that intercalates DNA, binds metal

ions, and reacts with oxygen to release ROS, creating oxidative base damage and DSB (32). Its effects may therefore be potentiated by higher ROS levels in PAH cells. Etoposide is a topoisomerase-II inhibitor that predominantly induces DSB but also generates ROS (33, 34), whereas griseofulvin induces aneuploidy. There is growing evidence that exposure to chemotherapeutic agents or ionizing radiation may cause pulmonary hypertension. Pulmonary vasculopathy may be a rare manifestation of graft-versus-host disease after allogeneic stem cell transplantation (35, 36), but there are also multiple reports of PAH or pulmonary venoocclusive disease that occurred in the absence of graft-versus-host disease, either in patients who received chemotherapy or radiotherapy alone, or after autologous stem cell transplantation (37–43).

These cases support a possible role for DNA damage in the pathogenesis of PAH

and suggest that it may occasionally be triggered by chemotherapy or radiotherapy in individuals who are unusually susceptible. Further studies are required to fully characterize the sensitivity of PAH cells to other exogenous and endogenous mutagens and the effectiveness of other antioxidants, but our results could have important implications for clinical management of patients in relation to procedures and treatments that involve radiation or induce DNA damage.

Several other questions remain to be answered, including the nature of genetic and environmental factors that lead to increased ROS production and DNA damage, both in health and in disease states. A recent study showed that release of hydrogen peroxide by LCL is genetically controlled and suggested an association with SNPs close to redox-related genes (44). Other candidate genes include the NADP reduced oxidase subunits and superoxide dismutases, as well as genes implicated in bleomycin sensitivity (45–47).

Of note, epigenetic down-regulation of superoxide dismutase-2 in the fawn-hooded rat leads to pulmonary hypertension (27). Levels of micronuclei may also be heritable (48), but overall, little is known about how levels of ROS and DNA damage vary in the general population, despite their critical roles in cancer, lung and cardiovascular diseases, neurodegenerative conditions, and normal aging (49–53).

It is also important to determine if DNA repair pathways are normal in individuals with PAH. We showed that one-third of PAH PAEC are chromosomally abnormal, a minimum estimate, because copy-neutral alterations, such as balanced translocations, are not detectable by SNP arrays. These abnormalities directly target genes that regulate cell proliferation, including *SMAD9*, one of the genes mutated in HPAH, and tumor suppressor genes, such as *RB1* and *BRCA2* (13). Interestingly, although increased DNA damage has been reported in PAH PASM (14), we did not observe a higher incidence of chromosomal changes than control PASM. Likewise, no abnormalities were detected in LCL or PBMC. This apparent difference in the persistence of chromosomal abnormalities in PAEC could indicate less efficient DNA repair and/or decreased likelihood for damaged cells to undergo apoptosis compared with other cell types. Even so, the number of abnormalities in PAEC is modest compared with the widespread genomic instability seen in cancer cells with DNA repair deficiency,

suggesting that DNA repair in PAH cells is largely normal.

PAH is hypothesized to be triggered by widespread endothelial cell death, followed by proliferation of apoptosis-resistant cells (54). In support of this, our previous studies, which included some of the PAEC now found to harbor chromosome abnormalities, showed that PAH PAEC are hyperproliferative and resistant to apoptosis (13, 55). In one case, we were able to show directly that a large interstitial deletion of chromosome 13 in PAEC conferred increased proliferation rate and altered bone morphogenetic protein signaling in comparison with chromosomally normal PASM from the same lung (13). The results of the present study therefore fit well with this hypothesis, which we now extend in Figure 6. Higher than average ROS production and DNA damage may constitute a risk factor for PAH by creating a pool of PAEC with genomic damage that are primed for hyperproliferation following endothelial injury (Figure 6). If the injury itself is genotoxic, our results suggest that PAH-susceptible cells have a heightened sensitivity to DNA-damaging agents that would further increase the incidence of genetic abnormalities. DNA damage may also promote cell survival and proliferation through PARP-1 activation (14). Then, as the disease progresses, additional mitochondrial, metabolic, and inflammatory changes could further increase the level of genotoxic stress,

leading to a vicious cycle of ongoing DNA damage and endothelial injury (Figure 6).

Our model unifies IPAH, HPAH, and APAH at the level of an underlying genetic predisposition to abnormal vascular remodeling, while recognizing that PAH is multifactorial and the causes of the cell damage that actually triggers the disease may be quite distinct between different types of PAH, for example shear stress in congenital heart defect PAH. Analysis of a much larger population of patients and their relatives in different PAH groups is needed to establish whether increased ROS production and DNA damage can be used as a biomarker for the risk of developing PAH and to determine if antioxidants might be a useful adjunct to existing therapy. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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