UltraRapid Communication

Microsatellite Instability of Endothelial Cell Growth and Apoptosis Genes Within Plexiform Lesions in Primary Pulmonary Hypertension

Michael E. Yeager, George R. Halley, Heiko A. Golpon, Norbert F. Voelkel, Rubin M. Tuder

Abstract—Primary pulmonary hypertension (PPH) is a frequently fatal disease whose pathobiology is poorly understood. Monoclonal endothelial cell growth is present within plexiform lesions of patients with PPH but not secondary PH because of congenital heart malformations. We hypothesized that endothelial cells within PPH plexiform lesions harbor mutations permissive for clonal cell growth. We found that endothelial cells in PPH plexiform lesions demonstrated microsatellite instability within the human MutS Homolog 2 gene (10 of 20 lesions) and displayed microsatellite site mutations and reduced protein expression of transforming growth factor-β receptor type II (6 of 19 lesions) and Bax (4 of 19 lesions). These results suggest that, in PPH, proliferated endothelial cells have genetic alterations associated with microsatellite instability and concomitant perturbation of growth and apoptosis gene expression akin to neoplasia. The full text of this article is available at http://www.circresaha.org. (Circ Res. 2001;88:e2-e11.)

Key Words: microsatellite instability ■ transforming growth factor-β receptor type II ■ Bax ■ primary pulmonary hypertension

Primary (idiopathic or unexplained) pulmonary hypertension (PPH) is a rare disease of precapillary pulmonary arterioles occurring in familial form, as a sporadic disease, or in the setting of anorexigen use or HIV infection. Severe secondary PH (2°PH), also characterized by precapillary endothelial cell proliferation, can occur due to congenital heart malformations or collagen vascular disorders. The pathobiology of PPH is incompletely understood in part due to lack of detailed knowledge of the natural history. However, patients with PPH always present with elevated pulmonary artery pressures and remodeling of the layers of the pulmonary vascular wall. In contrast to the obligatory endothelial cell monolayer in normal pulmonary arteries, lungs of patients with severe pulmonary hypertension demonstrate dysregulated intraluminal growth of endothelial cells, forming intravascular tumors (tumorlets) known as plexiform lesions. The endothelial cell growth eventually blocks the lumens of microscopic precapillary pulmonary arterioles, leading to fatal heart failure. Elucidation of the pathogenesis of these endothelial cell tumorlets may impact on current diagnostic and therapeutic modalities for severe pulmonary hypertension.

Endothelial cells within plexiform lesions of patients with PPH expand in a monoclonal fashion, ie, they arise from a single cell, whereas 2° PH lesions develop via a polyclonal expansion of endothelial cells. Because monoclonal cell growth has been consistently demonstrated in cancer and in smooth muscle cells within atherosclerotic plaques, our data establishing monoclonal endothelial cell growth in PPH challenge traditional concepts relating pulmonary vascular remodeling to vasoconstriction, vascular injury, or in situ thrombosis. We propose that endothelial cell proliferation in PPH displays mechanistic features common to neoplastic cell growth. More specifically, the finding of monoclonal cell growth implies that genetic mutation(s) may result in selective growth advantage of a single endothelial cell.

The transforming growth factor-β (TGF-β) family of signaling molecules inhibits proliferation of endothelial cells by modulating proteins involved in cell-cycle control and angiogenesis. Mutations in TGF-β signaling molecules have been implicated in initiation and progression of human cancers and atherosclerotic plaques. These mutations generally occur secondary to deficiencies in mismatch repair proteins such as hMSH2, leading to a mutator phenotype (MMP) and microsatellite instability (MSI). Specifically, frameshift mutations resulting from insertions or deletions within a 10 adenine microsatellite region in exon 3 of the TGF-β RI gene have been demonstrated in colon cancers and in smooth muscle cells from atherosclerotic plaques and restenotic lesions. Abnormal TGF-β signaling may confer a growth advantage to cells because loss of TGF-β facilitates tumor formation in transgenic mice.
Similarly, an 8-guanine exonic microsatellite region within exon 3 of Bax, a proapoptotic member of the Bcl-2 gene family, is prone to instability. This mutation, which occurs in >50% of MMP 1 colon adenocarcinomas, 47 confers a growth advantage to tumor cells via escape from apoptotic cell-death cascades involving Bax. 38

Because endothelial cells in plexiform lesions of patients with PPH grow in a monoclonal fashion, we investigated whether endothelial cells in PPH plexiform lesions demonstrate somatic mutations in microsatellite sites of key cell growth or apoptosis genes. We report herein that microdissected endothelial cell DNA harvested from plexiform lesions of patients with PPH, but not from 2P PH patients, exhibit MSI within hMSH2 and contain mutations in the TGF-β RII and Bax genes. Additionally, we show that endothelial cells within plexiform lesions of patients with PPH have reduced expression of TGF-β RII and Bax proteins. These data are, to our knowledge, the first in situ demonstration of somatic mutations within endothelial cells beyond the setting of neoplasia. Furthermore, our results suggest that these endothelial cell-proliferative vascular lesions could develop because of genetic instability, which may disrupt the physiologic quiescence of normal endothelial cells.

Materials and Methods

Case Selection

Formalin-fixed, paraffin-embedded blocks of lung and non-lung tissue (liver, kidney) from patients with PPH or 2P PH were retrieved from the archival collection of the Department of Pathology at the University of Colorado Health Sciences Center. Thirteen samples of PPH lungs (10 idiopathic, 2 associated with anorectic drug intake, 1 heart disease, 2 liver cirrhosis, 1 calcinosis, Raynaud’s, sclerodactyly, telangiectasia (CREST) syndrome, and 1 scleroderma) were obtained. Thirteen samples originated from autopsy material, 6 lungs were from lung transplantation, and 1 sample was from open lung biopsy. Laser-capture microdissection was carried out with a Pix Cell II LCM unit (Arcturus Engineering Inc) according to manufacturer’s protocols. The average number of 50- to 300-μm-diameter-sized fields microdissected from normal and PH patient tissues was 7, taken from 10-μm-thick serial sections (~100 cells/section). Only the core of the lesion was microdissected, avoiding smooth muscle cell contamination (Figure 1).

Analysis of Mutations

DNA Extraction

Twenty μL of proteinase K buffer and 2 μL proteinase K at 20 mg/mL were added to microdissected tissue followed by overnight incubation at 37°C. The enzyme was inactivated at 98°C for 8 minutes. The mixture was microcentrifuged for 1 minute at 15 000g.

Whole-Genome Amplification (WGA)

Microdissected cells were processed and amplified using polymerase chain reaction (PCR) as described previously using a High-Fidelity Long Template PCR kit (Boehringer Mannheim/Roche), 39 which, in combination with nested PCR, allows for successful amplification of single tumor-cell DNA. The reaction mixture was added to 10 μL microdissected DNA; total final volume was 60 μL. Exactly 5 μL of WGA PCR was used as a template for subsequent gene-specific PCR reactions. Additionally, each PCR reaction was run in parallel with DNA from 3 control cell lines. All samples demonstrated reproducibility in duplicate independent PCR runs.

TGF-β Receptor Type II (RII) PCR

PCR amplification for TGF-β RII was performed using high-fidelity Pwo polymerase (Boehringer), primer sequences, and reaction conditions as described previously by Markowitz et al. 47 The size of the amplicon (73 bases for RII) allows for discrimination of single-base-pair additions or deletions after gel electrophoresis. The forward primer was 5’-end-labeled using T4 polyadenylate kinase (PNK) (Promega) and γ-P32 ATP at 37°C for 30 minutes. Proteinase K was inactivated for 10 minutes at 70°C. Exactly 5 μL of WGA was used as a template for TGF-β RII PCR reactions. Control cells used were Hec1A, an endometrial adenocarcinoma that is wild-type homozygous at the TGF-β RII A9/A9 microsatellite; RL-95-2, an endometrial adenocarcinoma that is heterozygous A9/A9; and HCT116, a colon carcinoma that is homozygous A9. DNA extracted from normal lung was also used as a control, because microdissection of normal endothelial cells exclusively from a monolayer was technically unfeasible.

Bax PCR

PCR amplification was performed with primers producing a 94-base amplicon encompassing an 8-guanine microsatellite located in exon 3: 5’-ATCCAGGATCGACAGGCG CC-3’ and 5’-ACTCGGCTACGCTTCTTGTTG-3’. The reaction was prepared with 200 μM dNTP, 0.6 μM each primer, 10× buffer, and 2.5 U Pwo polymerase. This mixture was added to 5 μL of WGA product; total volume was 50 μL. PCR cycling conditions were as follows: 94°C 3 minutes, 94°C 30 seconds, 58°C 30 seconds, and 72°C 40 seconds, 30 cycles, and 72°C 4 minutes. Controls used were DNA isolated from normal whole lung and from control cells (wild-type Hec1A and heterozygous RL-95-2 and HCT116).

Electrophoresis

Sequencing gels of 6% polyacrylamide, 7 mol/L urea were used for Southern blots and mutational analyses. Five μL of loading buffer (95% formamide, 0.05% xylene cyanol, and 0.05% bromophenol blue) was added to PCR samples. Ten μL of sample mixture was electrophoresed 100 minutes at 90 W of constant power. The gel was vacuum-dried at 80°C for 70 minutes, exposed 12 hours to a phosphor storage screen, and analyzed with a Molecular Dynamics 860 STORM PhosphorImager using ImageQuant 5.0 software (Molecular Dynamics).

Densitometric Quantification of TGF-β RII PCR Band Intensities

To improve our ability to accurately assess the status of PH cases as wild type (+/+), heterozygous (+/−), or homozygous deleted (−/−), we measured TGF-β RII PCR band intensities using ImageQuant 5.0. Near the expected PCR products, there are shadow bands that may represent primer-dimer products and/or polymerase exonuclease activity. Therefore, we established average upper and lower band intensities for Hec1A, RL-95-2, and HCT116 control cells using the means of 6 separate experiments. The upper band was divided by the lower band to derive an intensity ratio with which 95% confidence intervals were established for wild-type, heterozygous-deleted, and homozygous-deleted phenotypes. PH and normal lung samples were PCR-amplified, electrophoresed, and measured by densitometry to quantify a band ratio between upper and lower gel bands. Using the results of densitometry, we assigned cases as +/+ , +/− , or −/− based on the comparison of ratios established for control cells. Heterozygous- and homozygous-deleted PCR bands were confirmed by Southern blot analysis and DNA sequencing. Densitometry was not performed for Bax or hMSH2 PCR products because shadow banding was minimal and separation of PCR products was clearly visible after gel electrophoresis.

TGF-β RII Southern Hybridization

PCR products were amplified (without radiolabel) and electrophoresed as described above, then transferred from sequencing gels onto a Zeta-Probe OT-positive charge nylon membrane (BIO-RAD) at 500 mA for 5 hours in 1× SSPE buffer. The hybridizing probe sequence used for TGF-β RII was 5’-CTCCAAAGTGCATTA-
Clinical Data of Patients With Severe Pulmonary Hypertension

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender/Age</th>
<th>PAP s/d/m</th>
<th>CO</th>
<th>Underlying Condition</th>
<th>Final Diagnosis</th>
<th>PG(_{II})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>Anorectic drug</td>
<td>PPH</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>F/53</td>
<td>107/47/29</td>
<td>2.2</td>
<td>Anorectic drug</td>
<td>PPH</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>F/50</td>
<td>133/44/NA</td>
<td>NA</td>
<td>Anorectic drug</td>
<td>PPH</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>HIV</td>
<td>PPH</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>F/37</td>
<td>115/50/70</td>
<td>2.9</td>
<td>None</td>
<td>PPH</td>
<td>No</td>
</tr>
<tr>
<td>6</td>
<td>F/37</td>
<td>85/37/59</td>
<td>3.2</td>
<td>None</td>
<td>PPH</td>
<td>No</td>
</tr>
<tr>
<td>7</td>
<td>M/26</td>
<td>87/57/73</td>
<td>3.4</td>
<td>None</td>
<td>PPH</td>
<td>Yes</td>
</tr>
<tr>
<td>8</td>
<td>M/53</td>
<td>106/38/62</td>
<td>4.8</td>
<td>None</td>
<td>PPH</td>
<td>Yes</td>
</tr>
<tr>
<td>9</td>
<td>F/30</td>
<td>100/42/61</td>
<td>2.7</td>
<td>None</td>
<td>PPH</td>
<td>No</td>
</tr>
<tr>
<td>10</td>
<td>F/48</td>
<td>180/80/112</td>
<td>1.9</td>
<td>None</td>
<td>PPH</td>
<td>No</td>
</tr>
<tr>
<td>11</td>
<td>M/40</td>
<td>96/50/65</td>
<td>2.1</td>
<td>None</td>
<td>PPH</td>
<td>NA</td>
</tr>
<tr>
<td>12</td>
<td>F/40</td>
<td>114/42/NA</td>
<td>NA</td>
<td>None</td>
<td>PPH</td>
<td>NA</td>
</tr>
<tr>
<td>13</td>
<td>F/30</td>
<td>79/63/70</td>
<td>2.2</td>
<td>None</td>
<td>PPH</td>
<td>Yes</td>
</tr>
<tr>
<td>14</td>
<td>F/60</td>
<td>NA</td>
<td>NA</td>
<td>Cholangiocarcinoma/biliary cirrhosis</td>
<td>2(^{*})PH</td>
<td>No</td>
</tr>
<tr>
<td>15</td>
<td>F/21</td>
<td>130/50/78</td>
<td>NA</td>
<td>CHD (SV, TGV)</td>
<td>2(^{*})PH</td>
<td>No</td>
</tr>
<tr>
<td>16</td>
<td>F/60</td>
<td>90(\ast)</td>
<td>NA</td>
<td>CREST</td>
<td>2(^{*})PH</td>
<td>No</td>
</tr>
<tr>
<td>17</td>
<td>M/51</td>
<td>NA</td>
<td>NA</td>
<td>Cirrhosis</td>
<td>2(^{*})PH</td>
<td>No</td>
</tr>
<tr>
<td>18</td>
<td>F/NA</td>
<td>NA</td>
<td>NA</td>
<td>Scleroderma</td>
<td>2(^{*})PH</td>
<td>NA</td>
</tr>
<tr>
<td>19</td>
<td>F/47</td>
<td>NA</td>
<td>NA</td>
<td>SVD</td>
<td>2(^{*})PH</td>
<td>NA</td>
</tr>
<tr>
<td>20</td>
<td>M/31</td>
<td>133/84/105</td>
<td>2.54</td>
<td>ASD</td>
<td>2(^{*})PH</td>
<td>NA</td>
</tr>
</tbody>
</table>

**DNA Dilution Experiments**

Artifact bands may arise from nested PCR amplification of minute amounts of template DNA. Therefore, we performed a screening of MSI, TGF-\(\beta\) RII, and Bax mutations. DNA from HUVECs (wild-type \([A_0]\) TGF-\(\beta\) RII, MSI\(^{-}\) and RL-95-2 \(+/−\) \([A_0]/A_0\) TGF-\(\beta\) RII, MSI\(^{-}\)) control cells was extracted, quantified, and serially diluted from a starting concentration of 0.1 \(\mu\)g/\(\mu\)L. Similarly, normal and PPH lung alveolar septa (not containing plexiform lesions) were harvested by laser microdissection using a range of between 1 to 9 fields to recapitulate a similar range taken from plexiform lesions. Nestcd PCR was performed as described above using identical amounts of DNA into WGA and constant (5 \(\mu\)L) amounts of WGA reaction into second PCR reactions.

**Quantification of Genomic DNA Using Quantitative PCR**

Concentrations of microdissected DNA were determined by quantitative PCR of the \(\beta\)-actin gene. Direct real-time detection of PCR product was monitored with the Gene Amp 5700 sequence detection system (Perkin-Elmer) measuring increasing fluorescence caused by binding of SYBR Green to double-stranded DNA. The forward primer sequence for \(\beta\)-actin was 5'-CCTGGACCCAGCACAAT-3'; the reverse primer sequence was 5'-GGGCGGACTCTGCTACA-TAC-3'. Five microliters of genomic DNA mixture was the template for a 50-\(\mu\)L PCR amplification using 10 \(\mu\)mol/L each of sense and antisense primers. The reaction mixture was as follows: 5 \(\mu\)L SYBR PCR buffer, 500 \(\mu\)mol/L dNTPs, 5.5 \(\mu\)mol/L MgCl\(_2\), and 5 U AmpliTaq Gold Polymerase (Perkin-Elmer). Polymerase was activated by incubating reactions at 95°C for 10 minutes followed by 40 cycles of amplification (95°C for 15 seconds and 60°C for 1 minute). The presence of specific amplification was confirmed by generating melting curve profiles after PCR. Additionally, we analyzed PCR products by agarose gel electrophoresis and confirmed specificity by cloning and sequencing. For absolute quantitation, we used plasmids (pGEM-T vector systems, JM109 cells; Promega) containing a cloned \(\beta\)-actin sequence in concentrations ranging from 0.5 to TGAAGG-3', beginning at 153 bp into exon 3, and ending at 174 bp into exon 3. The oligonucleotide probe was radiolabeled with PNK, and membranes were prehybridized with blocking solution containing 100 \(\mu\)g/mL sheared, denatured DNA for 1 hour at 49°C. Labeled probe was hybridized for 6 hours at 49°C and washed twice at 49°C in 2× SSPE buffer to remove background counts.

**Direct DNA Sequencing**

For confirmation of genetically unstable DNA, direct sequencing is advantageous over bacterial subcloning and sequencing due to possible misincorporation of nucleotides by bacterial polymerases during plasmid replication. PCR products were amplified without radiolabel as described above. Reactions were mixed with 6× loading buffer, run on a 2% agarose gel, and stained 20 minutes with ethidium bromide at 1 mg/mL. Bands were visualized using UV illumination, excised, transferred to a Gene Capsule (Geno Technology, Inc) electrophoretion chamber, placed in a mini gel electrophoresis box, and eluted 45 minutes at 90 V in TBE buffer. DNA was pipetted out at a final volume of 60 \(\mu\)L. Thirty microliters of excess eluate was evaporated off using a Sorvant SpeedVac concentrator, and samples were sequenced using an ABI 310 sequencer (Perkin-Elmer).
plexiform lesions demonstrated MSI tested were wild type. We found that 10 of 20 (50%) PPH analysis on BAT-26 (Figure 2A). All normal lungs (10 of 10) using the Vectastain Universal Quick kit (Vector Laboratories, Inc). Hydrogen peroxide for 15 minutes. Antibody staining was performed retrieving, endogenous peroxidase activity was blocked with 3% with known actin copy numbers. Plasmid and microdissected genomic DNAs were run in duplicate, and reactions without DNA were used to establish baseline levels for fluorescence.

Immunohistochemistry
Formalin-fixed, paraffin-embedded tissues were sectioned at 5 μm using a Leitz 1512 microtome. Sections were deparaffinized for 4 hours at 60°C, then rehydrated through xylene, 100% ethanol, and 95% ethanol washes. Sections were placed into a Nordicware pressure cooker containing 800 mL 1× sodium citrate/citric acid buffer and microwaved at 650 W for 18 minutes. After antigen retrieval, endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 minutes. Antibody staining was performed using the Vectastain Universal Quick kit (Vector Laboratories, Inc). Polyclonal antibodies recognizing TGF-β RII, and Bax (P-19, Santa Cruz Biotech Inc) or Bax (P-19, Santa Cruz) were diluted to concentrations between 1:50 and 1:100 and incubated on sections for 30 minutes at 25°C.

Results
Clinical data of patients with PPH and 2 PH are shown in the Table. Plexiform lesions occur in pulmonary precapillary arterioles measuring 50 to 300 μm in diameter; therefore, we harvested endothelial cell DNA by laser-capture microdissection to screen for mutations at microsatellite sites (Figures 1A and 1B). Subsequently, we performed WGA as the first of two PCR reactions (Figure 1C). Using an identical size aliquot of WGA as a template for nested PCR reactions allowed for parallel analysis of hMSH2, TGF-β RII, and Bax genes from a singular source of endothelial cells collected from plexiform lesions while simultaneously controlling DNA input into PCR reactions.

Endothelial Cells Within PPH Plexiform Lesions Are Genetically Unstable
Mutations within microsatellite sites have been associated with loss of repair enzymes such as hMSH2, which itself contains a microsatellite region (BAT-26). The functional significance of variations in BAT-26 microsatellite length is unclear, yet deviation from wild-type microsatellite length in BAT-26 (26 adenines) is consistent with generalized microsatellite instability (MSI). And a mutator phenotype (MMP). To assess whether endothelial cells within plexiform lesions were associated with MSI, we carried out PCR analysis on BAT-26 (Figure 2A). All normal lungs (10 of 10) tested were wild type. We found that 10 of 20 (50%) PPH plexiform lesions demonstrated MSI, whereas none (0 of 8) of the 2 PH plexiform lesions associated with congenital heart malformations demonstrated MSI. Figure 2B presents typical sequence data (total of 10 lesions from 6 patients with PPH) for BAT-26 PCR products, validating results of PCR analysis. Whole lung and liver or kidney DNA from patients with PPH or 2 PH, ranging from 1 microdissected field to an entire 10-μm section, demonstrated normal BAT-26 microsatellite length. Thus, allelic shifts detected within DNA from plexiform lesion endothelial cells did not result from polymorphism, germline MSI, or PCR artifact.

Endothelial Cells Within PPH Plexiform Lesions Contain TGF-β RII Mutations
Because TGF-β RII microsatellite mutations occur at a rate of ~90% in MSI colon cancers and in atherosclerotic lesions, we screened for these mutations in plexiform lesions (Figure 3A). All normal lungs (10 of 10) were wild type. In contrast, PPH plexiform lesions migrated as wild type (+/+), heterozygous one-base-deleted (+/−), or homozygous one-base-deleted (−/−) bands similar to results obtained with DNA prepared from 3 control (+/+, +/−, −/−) cell lines. We found that 6 of 19 (32%) PH plexiform lesions, but none of the 2 PH plexiform lesions (0 of 8 lesions), showed mutation of at least one allele within the TGF-β RII exon 3 microsatellite. Liver or kidney DNA from patients with PPH was wild type for TGF-β RII (0 of 8 patients).

We validated specificity of PCR bands by performing Southern blot analysis using a labeled internal probe to the TGF-β RII amplicon (Figure 3B). We also expressed results of TGF-β RII PCR amplification as the ratio of densitometric
band intensities of the upper 73-bp band and the lower 72-bp band (Figure 3C). This assisted us in initially classifying plexiform lesions as wild type, heterozygous, or homozygous for the TGF-βRII A10 microsatellite, because nonspecific shadow bands occur presumably attributable to exonuclease activity of the high-fidelity Pwo polymerase. In PPH lungs, densitometry of 16 microdissected plexiform lesions confirmed either a single-copy (4 of 16) or double-copy (1/16) mutation.

Using direct DNA sequencing, we corroborated results of the PCR, Southern blot, and densitometric analyses. The 72-bp product resulted from adenine loss within exon 3 (Figure 3D), not from truncation or mispriming of PCR amplicons. DNA sequence data were 100% consistent (17 lesions from 6 patients with PPH) with results obtained by TGF-βRII PCR, densitometry, and Southern blot mutational analyses.

Frameshift mutation imparted by loss of an adenine within the TGF-βRII microsatellite generates a stop codon 100 bases downstream and may result in translation of a truncated protein. Therefore, we analyzed TGF-βRII protein expression in PPH, 2′PH, and normal human tissue by immunohistochemistry (Figure 4). Using a polyclonal antibody recognizing wild-type TGF-βRII, we analyzed 35 plexiform lesions from 6 PPH lungs. Thirty-one of 35 (89%) PPH plexiform lesions did not express TGF-βRII, whereas endothelial cells within PPH pulmonary arteries not containing plexiform lesions and pulmonary arteries in 4 normal lungs expressed the receptor abundantly. Two of 27 (8%) of 2′PH plexiform lesions did not express TGF-βRII. The observed cytoplasmic pattern of TGF-βRII staining is consistent with previous immunohistochemical analyses using the identical antibody. These data are consistent with our PCR mutational analyses demonstrating a significant proportion of plexiform lesions in patients with PPH containing genetic mutations. Our results suggest that TGF-βRII microsatellite mutation is associated with absent or markedly decreased TGF-βRII protein expression.

Endothelial Cells Within PPH Plexiform Lesions Contain Bax Mutations

Because of the high incidence (~50%) of Bax microsatellite mutations in colon cancer, and our detection of MSI in plexiform lesions, we investigated whether patients with PPH harbored microsatellite mutations in Bax (Figure 5A). All normal whole-lung DNA tested was wild type. We found that 4 of 19 (21%) PPH plexiform lesions had microsatellite mutations. The shadow 72-bp band in normal lung samples does not shift significantly the ratio (mean = 3.7 ± 0.25 SE) when compared with that of the wild-type control Hec1A cell line (mean = 4.36 ± 0.44 SE). One plexiform lesion was homozygous, and 4 lesions have a heterozygous ratio. TGF-βRII mutation in these lesions was confirmed by DNA sequencing. D, Sequence analysis for wild-type TGF-βRII product (10 adenines) obtained from a normal lung and of a PPH plexiform lesion homozygous for a single base deletion (9 adenines). Note that microdissection of monolayer endothelial cells was technically unfeasible, so normal whole-lung DNA obtained from 10-μm sections was used as a wild-type control.
mutations in Bax, whereas none (0 of 8) of the 2P PHplexiform lesions showed Bax mutation. Interestingly, Bax mutation was seen only in patients with PPH that had taken anorexigen drugs, and all Bax mutations detected were heterozygous (1/2).

Using immunohistochemistry with a polyclonal antibody recognizing the N-terminus of Bax, we found that 18 of 28 (64%) PPH plexiform lesions stained positively for Bax, whereas 10 of 28 (36%) lesions were negative for Bax protein expression (Figures 5B through 5G). Pulmonary arterial endothelial cells in normal lungs, 2P PH plexiform lesions, or those arteries in PPH lungs not containing plexiform lesions showed a normal cytoplasmic pattern of Bax protein expression. The pattern of Bax staining we observed is consistent with previous immunohistochemical analysis using the identical antibody.42

We compared the common occurrence of MSI and mutations in TGF-β RII and/or Bax in DNA samples from 20 PPH plexiform lesions. When all 3 genes were successfully amplified (total of 13 lesions), the observed mutations in TGF-β RII and Bax occurred chiefly in the setting of MSI (Figure 6A). When 2 of 3 genes were successfully amplified, sporadic mutation in TGF-β RII occurred in the absence of MSI (1 of 2 lesions, Figure 6B). Conversely, a small proportion of MSI lesions developed without mutations in Bax (2 of 4 lesions, Figure 6C).

**Low Amounts of Input DNA Do Not Generate False-Positive Mutations**

Nested PCR amplification of low amounts of DNA harvested from plexiform lesions might have generated false-positive mutations. Therefore, we performed experiments using both a log dilution range of control-cell DNA and DNA from normal and PPH lungs obtained from microdissection of 1 to 9 microscopic fields containing alveolar septa. This field range is similar to that dissected from plexiform lesions for mutational studies. No spurious bands were generated for BAT-26 or TGF-β RII–nested PCR amplicons (Figures 7A and 7B). Additionally, we determined copy numbers of DNA present in microdissected samples shown in Figure 7 ranging from an equivalent of 539 (1 field) to 1530 (9 fields) copies of β-actin (Figure 8).
In the present study, we demonstrate, for the first time, the presence of genetic mutations within phenotypically abnormal, proliferative endothelial cells outside the setting of neoplasia. We examined whether genetic mutations are present within plexiform lesion endothelial cells in lungs from patients with PPH and 2ry PH using microdissection followed by nested PCR. Our results suggest that endothelial cells within plexiform lesions of patients with PPH acquire somatic mutations in genes whose protein products are involved in endothelial cell growth and death. Liver and kidney DNA or DNA from alveolar septa from patients with PPH did not show MSI or microsatellite mutation. Therefore, mutations in endothelial cells within plexiform lesions of patients with PPH did not result from germline mutation or polymorphism. These data bring into question the assumption that plexiform lesions represent an end-stage scar, resulting from vasoconstriction and/or increased shear stress.

Our finding of a high number of PPH plexiform lesions demonstrating microsatellite instability and gene mutations mirrors previous reports showing close association between microsatellite mutations in TGF-β RII and Bax and the microsatellite mutator phenotype (MMP). In our studies, MSI was assessed by PCR amplification of BAT-26, which has an incidence of polymorphism of 0.08% in Caucasians, and 7.7% in African Americans, and correlates 99.4% with larger panels of MSI markers. As proof of concept, we found the majority of MSI+ plexiform lesions also showed mutations in TGF-β RII, Bax, or both. Because the major mechanism of reduced expression of DNA mismatch repair proteins involves epigenetic gene silencing, the number of MSI+ patients with PPH may be higher.

Microsatellite mutations in endothelial cells from PPH plexiform lesions occur in exonic portions of TGF-β RII and Bax genes. These single-base deletions cause frameshift mutations within coding microsatellite sites, resulting in lack or marked reduction of functional protein expression. We cannot rule out the possibility that observed differences in the proportion of wild-type to mutated bands were caused by contamination with non–endothelial cell DNA during microdissection, yet this is unlikely due to precise, conservative harvesting of endothelial cells by laser-capture microdissection. The observed mutations were not the consequence of inaccurate PCR, because we used the high-fidelity Pwo polymerase, and most importantly, all normal whole-lung DNA consistently demonstrated wild-type length sequence. Additionally, we performed experiments using diluted control cell-line DNA and DNA from alveolar septal cells from normal and PPH tissues. No anomalies in microsatellite length could be detected in DNA concentrations equivalent to or below-average DNA concentrations extracted from microdissected plexiform lesions in our study. Results from PCR, densitometry, Southern blotting, direct DNA sequencing, and DNA dilution experiments were in complete concordance;

**Figure 6.** Venn diagrams demonstrating the concomitance of BAT-26, TGF-β RII, and/or Bax microsatellite mutations. A, Data obtained when all 3 genes were successfully amplified (total of 13 lesions). Three of 13 lesions showed MSI only, whereas five of 13 showed MSI and genetic mutation in TGF-β RII or Bax. Five of 13 lesions did not show MSI or mutation. B, Data from lesions that had only BAT-26 and TGF-β RII amplified (total of 2 lesions). One lesion showed mutation in TGF-β RII without MSI. C, Data from lesions that had only BAT-26 and Bax gene amplification (total of 4 lesions). Two lesions showed MSI without Bax mutation.

**Discussion**

In the present study, we demonstrate, for the first time, the presence of genetic mutations within phenotypically abnormal, proliferative endothelial cells outside the setting of neoplasia. We examined whether genetic mutations are present within plexiform lesion endothelial cells in lungs from patients with PPH and 2ry PH using microdissection followed by nested PCR. Our results suggest that endothelial cells within plexiform lesions of patients with PPH acquire somatic mutations in genes whose protein products are involved in endothelial cell growth and death. Liver and kidney DNA or DNA from alveolar septa from patients with PPH did not show MSI or microsatellite mutation. Therefore, mutations in endothelial cells within plexiform lesions of patients with PPH did not result from germline mutation or polymorphism. These data bring into question the assumption that plexiform lesions represent an end-stage scar, resulting from vasoconstriction and/or increased shear stress.

Our finding of a high number of PPH plexiform lesions demonstrating microsatellite instability and gene mutations mirrors previous reports showing close association between microsatellite mutations in TGF-β RII and Bax and the microsatellite mutator phenotype (MMP). In our studies, MSI was assessed by PCR amplification of BAT-26, which has an incidence of polymorphism of 0.08% in Caucasians, and 7.7% in African Americans, and correlates 99.4% with larger panels of MSI markers. As proof of concept, we found the majority of MSI+ plexiform lesions also showed mutations in TGF-β RII, Bax, or both. Because the major mechanism of reduced expression of DNA mismatch repair proteins involves epigenetic gene silencing, the number of MSI+ patients with PPH may be higher.

Microsatellite mutations in endothelial cells from PPH plexiform lesions occur in exonic portions of TGF-β RII and Bax genes. These single-base deletions cause frameshift mutations within coding microsatellite sites, resulting in lack or marked reduction of functional protein expression. We cannot rule out the possibility that observed differences in the proportion of wild-type to mutated bands were caused by contamination with non–endothelial cell DNA during microdissection, yet this is unlikely due to precise, conservative harvesting of endothelial cells by laser-capture microdissection. The observed mutations were not the consequence of inaccurate PCR, because we used the high-fidelity Pwo polymerase, and most importantly, all normal whole-lung DNA consistently demonstrated wild-type length sequence. Additionally, we performed experiments using diluted control cell-line DNA and DNA from alveolar septal cells from normal and PPH tissues. No anomalies in microsatellite length could be detected in DNA concentrations equivalent to or below-average DNA concentrations extracted from microdissected plexiform lesions in our study. Results from PCR, densitometry, Southern blotting, direct DNA sequencing, and DNA dilution experiments were in complete concordance;

**Figure 7.** Autoradiography of nested PCR amplicons for BAT-26 (A) and for TGF-β RII (B) using normal or PPH alveolar septal DNA obtained by laser microdissection of a range between 1 to 9 microdissected fields. All samples are of wild-type length (126 bp for BAT-26, 73 bp for TGF-β RII) compared with controls. *Average number of fields microdissected for plexiform lesions.
Therefore, low amounts of input DNA do not generate false-positive mutations in our assays.

We also show that TGF-β RII, an important growth regulatory gene, is mutated in a significant number of lesions and that this mutation is strongly associated with endothelial cell MSI in PPH. The discrepancy between 32% of plexiform lesions from patients with PPH harboring TGF-β RII DNA mutations and 89% of PPH plexiform lesions not expressing TGF-β RII protein by immunohistochemistry might be due to haploinsufficiency effects or dominant-negative inhibition. Loss of a single allele of TGF-β RII presumably results in 50% expression of wild-type TGF-β RII protein and consequently only ~25% of functional homodimeric receptor complexes at the cell surface. Alternatively, the truncated Aα receptor we detected in PPH plexiform lesions at the DNA level, if heterozygous in expression, could be acting as a dominant-negative as a result of a lack of C-terminal serine/threonine kinase activity. Either mechanism may impair endothelial cell TGF-β receptor signaling to insufficiently low levels permissive for clonal cell growth. Reduced expression of TGF-β signaling molecules in patients with PPH could also be due to epigenetic gene silencing, as has been demonstrated for mismatch repair enzymes. This phenomenon was not investigated in our experiments.

Therefore, for PPH, we propose a paradigm similar to colon cancer, in which a subset of patients exhibits a mutator phenotype caused by microsatellite instability. Loss of TGF-β RII is associated with progression from adenoma to carcinoma, and overexpression of wild-type TGF-β RII plasmids into TGF-β RII −/− colon carcinoma cells results in restoration of TGF-β −mediated growth inhibition. Recently, decreased RII/RI TGF-β receptor ratios were localized in smooth muscle cells of atherosclerotic plaques. Interestingly, genomic instability of TGF-β RII in these plaques suggests that monoclonal expansion of smooth muscle cells may be linked to loss of TGF-β RII. Indeed, impaired signaling of TGF-β family members has been identified in patients with hereditary hemorrhagic telangiectasia (endoglin and activin receptor-like kinase) and in familial PPH (bone morphogenetic protein receptor type II [BMPR-II]). These studies, combined with cell culture experiments, assign to the TGF-β signaling system the role of an important growth suppressor that, if impaired, leads to cell proliferation in cancer cells and vascular cells.

In addition to mutations in the growth-suppressive TGF-β RII, we show that an exonic microsatellite within the proapoptotic Bax gene is a mutational target in endothelial cells of PPH plexiform lesions. This frameshift mutation results in loss of the C-terminal BH3 transmembrane domain required for insertion of Bax into mitochondrial membranes and subsequent initiation of apoptotic cascades. Recently, Ionov et al showed that heterozygous instability at the Bax G8 microsatellite is sufficient to provide a selective growth advantage to colon cancer cells. These researchers took advantage of potential reversibility of microsatellite mutations. Bax microsatellite–mutated (+/−) and (−/−) HCT116 cells outgrew wild-type Bax HCT116 cells when injected into nude mice. We used an N-terminal–directed Bax antibody capable of detecting both wild-type and truncated Bax forms. Therefore, negative staining for 36% of plexiform lesions from patients with PPH may underestimate the true number of lesions deficient in functional Bax expression. These data strongly indicate that active selection of microsatellite instability within genes involved in regulation of cell growth and apoptosis is an important phenomenon in vivo, as seen in cultured endothelial cells.

Recently, patients with familial PPH were found to have heterozygous germline mutations in BMPR-II, a TGF-β family signaling receptor. In each study, DNA was prepared from peripheral blood, whereas we obtained DNA harvested directly from endothelial cells within plexiform lesions, the site of pathogenesis in pulmonary hypertension. Both groups invoke dominant-negative or haploinsufficiency models to explain the role of heterozygous mutant BMPR-II, yet the precise mechanism(s) by which mutant BMPR-II participates in the pathogenesis of pulmonary vascular remodeling in PPH has not been determined. The fact that not all patients with familial PPH have BMPR-II mutations and that such muta-
tions are heterogeneous implies that somatic genetic events do occur in lungs of familial patients with PPH.55–57 Whether plexiform lesions of familial patients with PPH demonstrate MSI or microsatellite mutations in TGF-β RII or Bax is unknown, although in preliminary experiments we have failed to detect any such mutations (M. Yeager, unpublished observations, 2000).

Although we cannot definitively conclude that genetic mutations cause pulmonary endothelial cell proliferation in humans, monoclonal expansion of endothelial cells in PPH may result from somatic microsatellite mutations in growth and death regulatory genes such as TGF-β RII and Bax. A second hit could take the form of hypoxia, viral infection, anorexin drug use, or additional genetic mutations.58 It is important to note that a putative endothelial stem-like cell has been described in humans, monoclonal expansion of endothelial cells in PPH.2000). In familial patients with PPH demonstrate MSI is an initiating event in these lesions, it could result from chronic inflammation present in perivascular sites in PPH lungs.59 Indeed, chronic intestinal inflammation in the setting of ulcerative colitis is associated with genetic instability and increased risk of colon cancer.35,48,60

Acknowledgments

This work was supported by National Institutes of Health grants 1RO1 HL60195-01 (to R.M.T.) and 1RO1 HL60913-01 (to N.F.V.), the American Heart Association, Desert Mountain Division Grant HL-00513532 (to R.M.T.), and the Shirley Kiner Witham Memorial Pulmonary Hypertension Research Fund. We thank the Sanford Markowitz Laboratory for the TGF-β RII PCR assay and the University of Colorado Health Sciences Center DNA Sequencing Core Laboratory for sequence analyses.

References


Microsatellite Instability of Endothelial Cell Growth and Apoptosis Genes Within Plexiform Lesions in Primary Pulmonary Hypertension

Michael E. Yeager, George R. Halley, Heiko A. Golpon, Norbert F. Voelkel and Rubin M. Tuder

_Circ Res._ 2001;88:e2-e11
doi: 10.1161/01.RES.88.1.e2

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/88/1/e2

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation Research_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation Research_ is online at:
http://circres.ahajournals.org/subscriptions/