Monoclonal Endothelial Cell Proliferation Is Present in Primary but not Secondary Pulmonary Hypertension

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Abstract

The etiology and pathogenesis of the vascular lesions characterizing primary pulmonary hypertension (PPH), an often fatal pulmonary vascular disease, are largely unknown. Plexiform lesions composed of proliferating endothelial cells occur in between 20 and 80% of the cases of this irreversible pulmonary vascular disease. Recently, technology to assess monoclonality has allowed the distinction between cellular proliferation present in neoplasms from that in reactive nonneoplastic tissue. To determine whether the endothelial cell proliferation in plexiform lesions in PPH is monoclonal or polyclonal, we assessed the methylation pattern of the human androgen receptor gene by PCR (HUMARA) in proliferated endothelial cells in plexiform lesions from female PPH patients ($n = 4$) compared with secondary pulmonary hypertension (PH) patients ($n = 4$). In PPH, 17 of 22 lesions (77%) were monoclonal. However, in secondary PH, all 19 lesions examined were polyclonal. Smooth muscle cell hyperplasia in pulmonary vessels ($n = 11$) in PPH and secondary PH was polyclonal in all but one of the examined vessels. The monoclonal expansion of endothelial cells provides the first marker that allows the distinction between primary and secondary PH. Our data of a frequent monoclonal endothelial cell proliferation in PPH suggests that a somatic genetic alteration similar to that present in neoplastic processes may be responsible for the pathogenesis of PPH. (*J. Clin. Invest.* 1998. 101:927–934.) Key words: pulmonary hypertension • endothelial cell • plexiform lesion • clonality • angiogenesis

Introduction

Primary or unexplained pulmonary hypertension (PPH),[1] an often fatal disease (1), is a diagnosis of exclusion. The disease exists in a familial form (2), and in sporadic occurrence. Clinically, the disease is characterized by elevated pulmonary artery pressure and progressive right heart failure (3). The median life expectancy after diagnosis is two or three years, with death usually due to right heart failure (1). The etiology and pathogenesis of the vascular alterations in PPH are largely unknown. The mapping of the gene for familial PPH, which accounts for 6% of the cases recorded in the National Institutes of Health PPH Registry (4), to the chromosome 2q31–32 (5) suggests that genetic predisposition plays a role in the development of the disease.

The plexiform lesion, which occurs in up to 80% of the patients with PPH (6), constitutes the hallmark of irreversible vessel disease in PPH (7). PPH patients with frequent plexiform lesions bear a worse prognosis than patients with pulmonary hypertension that is characterized histologically by the presence of thrombotic lesions (8). Our understanding of the genesis of the plexiform lesions remains rudimentary. However, we provided evidence recently which implicates proliferation of endothelial cells in this form of vascular remodeling (6, 9). The proliferation of endothelial cells in the plexiform lesions of patients with PPH and with secondary pulmonary hypertension (PH) (e.g., congenital heart disease with left to right shunt, collagen vascular disease, or HIV infection) (10) may be seen as a forme fruste of angiogenesis. Presently, it is unclear whether the plexiform lesion is pathogenically relevant or rather the result of progressive and end-stage scarring of pulmonary arteries in severe pulmonary hypertension.

A monoclonal cell population originates from a single cell which, because of a somatic mutation, has acquired growth advantage. Monoclonality represents the earliest event involved in the generation of neoplasms (11). Since normal or reactive hyperplastic tissues are usually composed of polyclonal cell populations, the presence of a monoclonal endothelial cell proliferation in PPH would support the concept that an acquired somatic mutation may be involved in the pathogenesis of the disease, particularly if monoclonality is not found in secondary PH. Because of our previous observations of unique endothelial cell clusters (tumorlets) in evolving plexiform lesions (6), we wondered whether there is a monoclonal endothelial cell proliferation in the plexiform lesions in PPH. Monoclonality has been demonstrated recently in Kaposi's sarcoma

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1. *Abbreviations used in this paper:* CREST, calcinosis, Raynaud's, sclerodactyly, telangiectasia; FAP, familial adenomatous polyposis; HUMARA, human androgen receptor gene methylation assay; PH, pulmonary hypertension; PPH, primary PH; STR, short tandem repeat.

(12), and clusters of genetically uniform smooth muscle cells and myofibroblasts were described in atherosclerotic plaques (13). In addition, the finding of a molecular marker for PPH may help us determine whether PPH represents a single homogenous disease, or alternatively, whether pulmonary hypertension related to dexfenfluramine, toxic oil syndrome, HIV infection, or hepatitis C is similar to PPH, rather than to secondary PH such as that caused by high pulmonary blood flow.

To assess the clonality of endothelial cell proliferation in plexiform lesions, we studied the pattern of X-chromosome methylation (14, 15) in the tissue from female patients with primary and secondary PH. At an early stage of embryo development, the activity of one of the two X-chromosomes in each cell is determined by differential methylation, which remains unchanged during subsequent somatic cell divisions. By using a genetic marker such as the human androgen receptor gene, which is invariably methylated in the inactive X-chromosome, it is possible to determine that in polyclonal tissues, approximately half of the cells have a methylated X-chromosome marker from one parent, and the remaining half have a methylated X-chromosome from the other parent. By contrast, a clonal cell expansion derived from a common progenitor cell exhibits a uniform pattern of X-chromosome methylation for a specific genetic marker. This principle can be demonstrated only in tissues from female subjects who are heterozygous for an X-linked marker gene that in normal tissue has an approximately balanced pattern of methylation. In this study, for the marker gene we used the X-linked human androgen receptor gene. The human androgen receptor gene is highly polymorphic (with a > 90% prevalence of heterozygosity) due to the presence of a CAG trinucleotide repeat sequence, which is 3′-prime of invariably methylated sites present in the promoter region for the human androgen gene locus in the inactive X-chromosome (16, 17).

Methods

Case materials

Formalin-fixed, paraffin-embedded blocks of lung tissue from female patients with PPH and secondary PH with plexiform lesions were retrieved from the archival collection of the Department of Pathology of the University of Colorado Health Sciences Center. One secondary PH case was provided by Dr. West Tyson (Children's Hospital, Denver, CO), and a second case by Dr. Martha Warnock (Department of Pathology, University of California, San Francisco). Six cases of PPH and five cases of secondary PH (four congenital heart disease with left to right shunt and one calcinosis, Raynaud's, sclerodactyly, telangiectasia [CREST] syndrome) were entered into the study. The clinical information is presented in Table I. Six cases originated from autopsy material (five PPH, one secondary PH); three lungs were obtained at the time of lung transplantation (one PPH, two secondary PH), and two lung specimens (secondary PH) were from open lung biopsy. Between 1 and 12 paraffin blocks (mean = 4 blocks) per patient were examined. From each tissue block, 50 serial tissue sections of 10-μm thickness were obtained. Plexiform lesions were mapped on hematoxylin and eosin–stained sections from every 6th slide. Only plexiform lesions of > 120 μm in length were selected for microdissection. One slide from each selected plexiform lesion was used for Factor VIII related antigen immunostaining. The remaining slides were eosin-stained for microdissection under a dissecting microscope at a magnification of 40. Cells representing the endothelial proliferation in plexiform lesion, the smooth muscle cells in medial hypertrophy of small pulmonary arteries, and lung parenchyma were microdissected with a 30-gauge needle, suspended in 100% glycerol, and aspirated with a hand-held glass micropipet under vacuum control (see Fig. 1) (18). Proliferating endothelial cells in each plexiform lesion were pooled from all eosin-stained slides with dissectable lesions. Smooth muscle cells from pulmonary arteries were pooled from 10 serial sections (100 μm in length). Control tissue consisted of adjacent lung parenchyma from each of the cases. We measured the diameter and length of the dissected pulmonary vessels in PPH and secondary PH for equal representation of the endothelial cells dissected. The amount of dissected tissue (long diameter × short diameter × length) from each plexiform lesion was $3.44 \pm 1.86 \times 10^{-3}$ mm^3 (mean±SD) (range 1.00–7.70) in the PPH group and $2.75 \pm 1.52 \times 10^{-3}$ mm^3 (range 0.84–6.50) in the secondary PH group. There was no statistical difference between the two groups. The plexiform lesions in PPH and secondary PH were morphologically indistinguishable.

Analysis of clonality

Clonality was determined with an adaptation of the human androgen receptor gene methylation assay (HUMARA) (17). The human androgen receptor gene, located to Xcen-q13, contains an in-frame short tandem repeat (STR) of the sequence (CAG)$_n$, where $n = 11$–31, and is polymorphic in $\sim$ 90% of cases (16, 17). The methylation status of the two HhaI sites located $\sim$ 100 bp upstream to the trinucleotide repeat has been shown to correlate with X-chromosome inactivation (17). Patterns of X-chromosome inactivation were determined by digestion of genomic DNA with methylation-sensitive restriction endonuclease HhaI, followed by PCR amplification of the polymorphic human androgen receptor STR, using a two step nested primer approach. This approach produces a high level of sensitivity and specificity of amplification of the human androgen receptor target, facilitating analysis of clonality in archival formalin-fixed tissues (19).

Processing of dissected tissue. The pooled tissue obtained by microdissection was suspended in 20 μl of proteinase K buffer (0.2 mg/ml proteinase K, 1 mM EDTA, 50 mM Tris-HCl, pH 8.4, 0.5% Tween). The suspensions were incubated overnight at 37°C, then inactivated by heat at 98°C for 8 min before their subsequent manipulation.

Restriction endonuclease HhaI digestion of active X-chromosome. For each sample, two reactions were prepared. In one, 10 μl of the DNA preparation mixture was subjected to 20 U HhaI (GIBCO BRL, Gaithersburg, MD) in 1.0× PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl$_2$; GIBCO BRL). In the other, 10 μl of the DNA preparation mixture was incubated with the buffer with no enzyme added. All reactions were 25 μl in total volume, and all incubations were carried out at 37°C for 12 h. After a second round of digestion performed with freshly added HhaI for 2 h, the reactions were terminated by incubating the mixture at 70°C for 20 min.

PCR. The first round of amplification was performed using outer primer 1 (5′-GCTGTGAAGGTTGCTGTTCCTCAT-3′) and primer 3 (5′-CGTCCAAGACCTACCGAGGAGCTT-3′) (19). The second round was performed with inner primer 2 (5′-TCCAGAATCTG-TTCCAGAGCGTGC-3′) and primer 4 (5′-ATGGGCTTGGG-GAGAACCATCCTC-3′) (19). The first round of PCR contained dNTP (50 μM), primers 1 and 3 (10 nM each), MgCl$_2$ (1.5 mM), Tris-HCl, pH 8.4 (20 mM), KCl (50 mM), *Taq* polymerase (0.25 U), 0.01% gelatin, and 10 μl of HhaI digest, in a total volume of 50 μl. After initial denaturation at 94°C for 3 min, PCR was performed using a thermal profile of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C, for a total of 32 cycles followed by a final extension of 10 min at 72°C. The second step PCR, using the same thermal profile, was performed with 1.0 μl of the first step PCR product using primers 2 and 4 (500 nM each), dNTP (200 μM), 0.1 μl of [α-^{32}P]deoxycytidine triphosphate (3,000 Ci/mmol), MgCl$_2$ (1.5 mM), KCl (50 mM), Tris-HCl, pH 8.4 (20 mM), *Taq* polymerase (0.25 U), and 0.01% gelatin, in a total volume of 50 μl. Each round of PCR used a hot start protocol with PCR gems (Perkin-Elmer Corp., Norwalk, CT).

Electrophoretic analysis of human androgen receptor gene STR amplification products. The PCR products were analyzed by electrophoresis on a 6% denaturing polyacrylamide sequencing gel and au-

toradiography (17). Quantitation of the PCR product bands was done by densitometric analysis using gel analysis software (SigmaGel®; Jandel Scientific Software, San Rafael, CA). Each allele is represented in the polyacrylamide gel by two major bands. Because internal [^{32}P]dCTP incorporation was used, both strands of DNA were detected, and the differential migration results from the fact that each has complementary base composition. The presence of lighter bands is assumed to occur due to the slippage of the polymerase during PCR. As shown, PCR products from HhaI-undigested template (lane HhaI−) from proliferated endothelial cells of plexiform lesion, smooth muscle cells of medial hypertrophy, and lung parenchyma yielded four major bands representing two alleles, one from the maternal and the other from the paternal parent (see Figs. 1–4). For each sample, the allele inactivation ratio was calculated by dividing the allele amplification ratio of the HhaI-digested sample (allele 1/allele 2) by the allele amplification ratio of the HhaI-nondigested sample (allele 1/allele 2) in order to correct for preferential amplification of one allele versus the other, as might occur if the two alleles differed markedly in the length of their repeats (20). The use of the normal tissue allele inactivation ratio corrects for the degree of an aberrant X-chromosome inactivation in normal tissue. A clonality ratio for each lesion was determined by dividing the allele inactivation ratio of the lesion by that of the lung parenchyma, thereby normalizing values for possible unequal lyonization (20). If the clonality ratio was > 1, it was substituted as its inverse. Analysis of cases was based on the assumption that if the lesion has balanced methylation between two alleles, suggestive of polyclonal composition, the clonality ratio should range between 0.25 and 1, and if it has completely nonrandom methylation, suggestive of monoclonal composition, the clonality ratio should range between 0 and 0.25. A clonality ratio < 0.25 was considered evidence of nonrandom methylation, as used in a previous study of clonality (12). A similar clonality ratio (0.3) was used in a different study (13).

Controls for analysis of clonality. DNA from the endometrial adenocarcinoma cell line HEC-1-A (American Type Culture Collection, Rockville, MD) was used as a positive control for restriction digestion and PCR amplification. This cell line is heterozygous at the human androgen receptor STR and shows complete disappearance of one allele band after restriction digestion (see Fig. 3). Reagent controls lacking target DNA were used as negative controls to monitor for contamination by PCR amplification products.

Results

Lung tissue from six patients with PPH and five patients with secondary PH (four associated with congenital heart disease with left to right shunt and one associated with the CREST syndrome) were initially available for the study. Two cases of PPH were excluded because PCR amplification of the DNA of lung tissue was not successful, and one case of congenital heart disease was excluded because of homozygosity for the human androgen receptor gene. PCR amplification of the lung DNA from all of the cases showed balanced methylation (i.e., almost even amplification) between the two human androgen receptor alleles after digestion with the restriction endonuclease HhaI (allele inactivation ratio = 1.09±0.20, mean±SD), thereby excluding the possibility of unequal lyonization (Table I).

Monoclonality of endothelial cell proliferation in the plexiform lesions in the lungs of PPH patients. Using microdissection, we obtained enriched populations of proliferated endothelial cells from each plexiform lesion (Figs. 1 and 2). 24 plexiform lesions from 4 PPH patients were assessed for clonality of the endothelial cell proliferation. Of the 24 plexiform lesions analyzed, PCR amplification was successful in 22. Unbalanced methylation (the predominance of one human androgen receptor allele after HhaI restriction digestion, i.e., clonality ratio < 0.25), diagnostic of monoclonal composition of the proliferated endothelial cells within the plexiform lesion, was found in 17 (77%) of these 22 lesions. 13 of 17 lesions showed complete knock-out of the band representative of one allele after the restriction digestion with HhaI (clonality ratio = 0). Clonality ratios ranging between 0 and 0.25 can be explained by the presence of a minor population of smooth muscle cells or inflammatory cells harvested with the endothelial cells during microdissection. The pattern of methylation of the human androgen receptor alleles of the proliferated endothelial cells in different plexiform lesions in a given female PPH patient was discordant in three of four patients. Overall, the smaller allele was methylated preferentially in 10 lesions, and

Table I. Selected Clinical and Clonality Data of Eleven Women with Primary or Secondary PH

Patient No.	Diagnosis	Age	Pulmonary arterial pressure*	Allele inactivation ratio‡	Number of plexiform lesions studied	Unbalanced methylation§
		yr	mmHg			
1	PPH	48	93/36/58	1.28	9	7
2	PPH	37	85/37/59	0.86	5	4
3	PPH	40	101/47/69	0.99	5 (3)‖	3
4	PPH	30	102/39/64	1.43	5	3
5	PPH	37	70/40/50	Not PCR-amplified		
6	PPH	39	111/50/70	Not PCR-amplified		
7	CHD (VSD)	24	Not available	1.18	3	0
8	CHD (PDA)	2	85/55/60	1.06	5	0
9	CHD (ASD)	32	125/50/78	0.91	8	0
10	CREST	53	40/15/28	0.97	3	0
11	CHD (ASD)	19	100/60/80	Homozygous HUMARA alleles		

*Systolic/diastolic/mean. ‡Of lung parenchyma (refer to Methods); the average allele inactivation ratio was 1.09, excluding the possibility of unequal lyonization. §Number of plexiform lesions with clonality ratio < 0.25 (refer to Methods), suggestive of monoclonality. ‖Of the five plexiform lesions analyzed, PCR amplification was successful in three. *CHD*, Congenital heart disease with left to right shunt. *ASD*, Atrial septal defect. *PDA*, Patent ductus arteriosus. *VSD*, ventricular septal defect. *HUMARA*, Human androgen receptor.
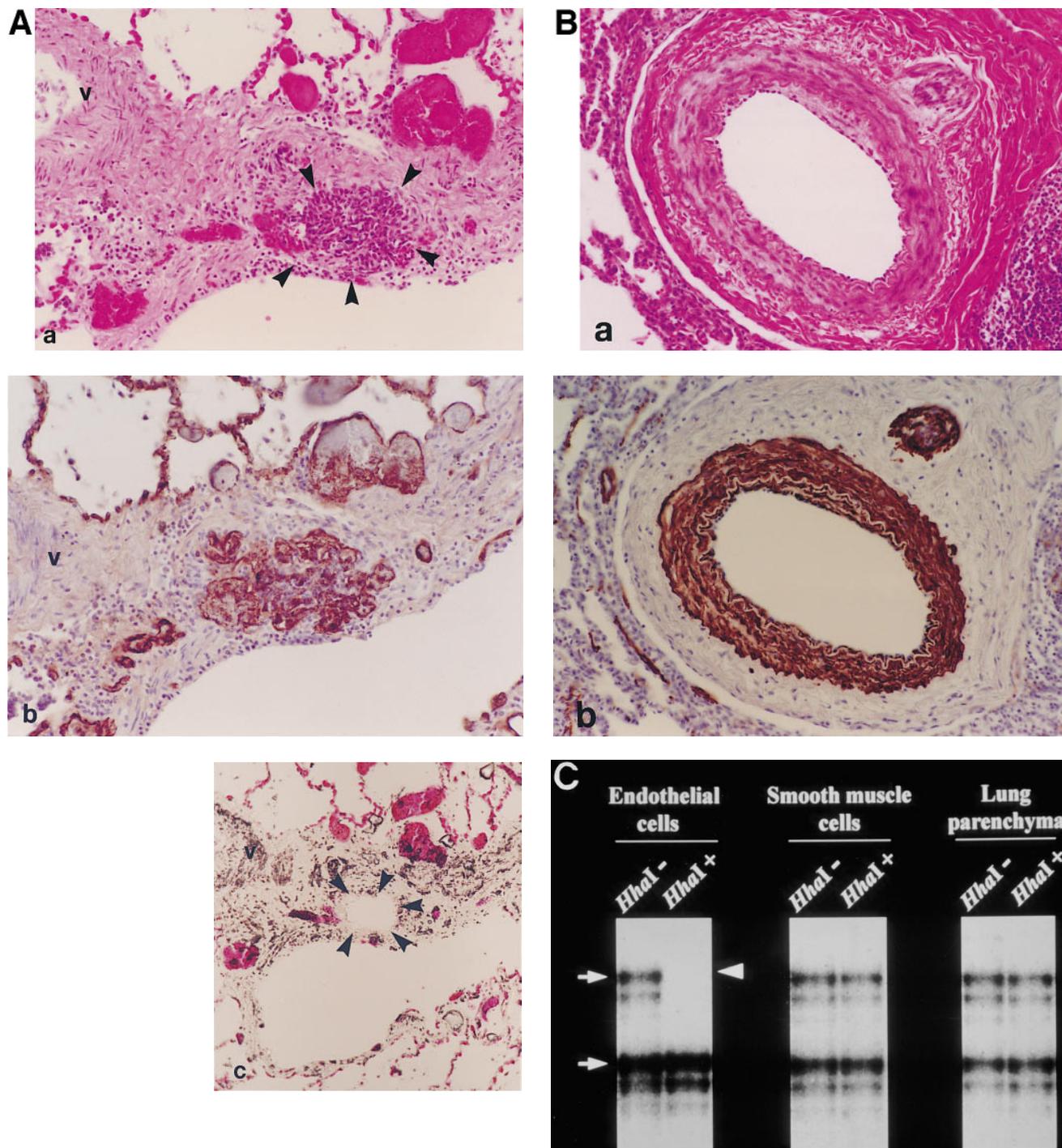
Figure 1. Assessment of clonality of endothelial cells in a plexiform lesion and of smooth muscle cells in PPH (patient 1). (*A*) Morphology of a plexiform lesion in PPH, showing (*a*) prominent intravascular proliferating endothelial cells (hematoxylin and eosin staining, ×200) (*arrowheads*), (*b*) intense immunostaining for Factor VIII related antigen (immunoperoxidase ×200), and (*c*) that the proliferating endothelial cells were harvested selectively by microdissection for subsequent PCR amplification (eosin staining, ×200) (*arrowheads*). *v,* Pulmonary artery. (*B*) Morphology of medial hypertrophy of small pulmonary artery (from patient 1), showing (*a*) prominent thickening of smooth muscle layer (hematoxylin and eosin staining, ×400) with (*b*) intense immunostaining for muscle-specific antigen (immunoperoxidase ×400). (*C*) Clonality analysis based on HUMARA of DNA from endothelial cells in a plexiform lesion (shown in *A*) and from vascular smooth muscle cells (of vessel shown in *B*) in PPH. Lane *HhaI*−, Sample without HhaI digestion; lane *HhaI*+, HhaI-digested sample. In proliferated endothelial cells from plexiform lesion, one parental allele (*arrowhead*) is absent after HhaI digestion (lane *HhaI*+) (clonality ratio = 0, refer to Methods), representative of unbalanced methylation pattern in the monoclonal population of cells. However, in the smooth muscle cells from medial vascular hypertrophy, two parental alleles are present after HhaI digestion (clonality ratio = 0.85), representative of balanced methylation pattern in the polyclonal population of the cells. In the lung parenchyma, both alleles are amplified equally after HhaI digestion, excluding the possibility of unequal lyonization of normal tissue.

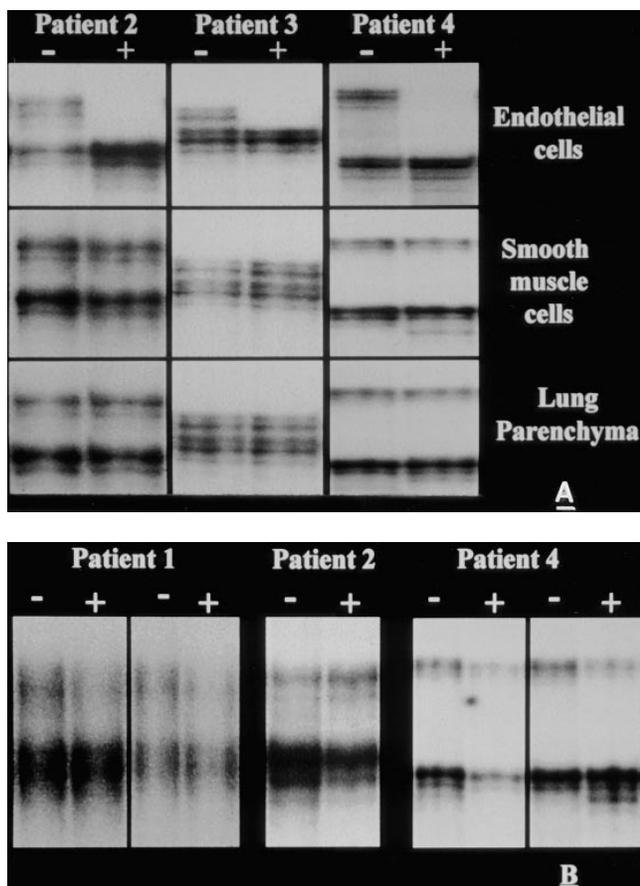*Figure 2.* (*A*) HUMARA of representative examples of monoclonal plexiform lesions, polyclonal pulmonary artery smooth muscle cell, and lung samples in PPH patients 2, 3, and 4. −, Without HhaI digestion; +, HhaI-digested sample. (*B*) HUMARA showing polyclonality of 5 of 22 plexiform lesions in PPH patients.−, Without HhaI digestion; +, HhaI-digested sample.

the larger allele predominated in the remaining 7. The clonality ratio of the proliferated endothelial cells of the 22 plexiform lesions was 0.15 ± 0.25 (mean$\pm$SD) (range 0–0.72) (Figs. 1, 2, and 5). The polyclonal lesions present in PPH patients 1, 2, and 4 are shown in Fig. 2 *B*.

Polyclonality of endothelial cell proliferation in the plexiform lesions in the lungs of patients with secondary PH. In 4 secondary PH patients, 19 plexiform lesions histologically similar to those in PPH lungs were examined to assess the clonality of the endothelial cell proliferation. HUMARA was successful in all of the lesions sampled. None of the 19 lesions showed unbalanced methylation, since the clonality ratio of all 19 lesions was 0.68 ± 0.19 (range 0.38–0.95) (Figs. 3–5), consistent with a polyclonal composition of the proliferated endothelial cells.

Clonal analysis of smooth muscle cells obtained from small pulmonary arteries characterized by medial hypertrophy. From both PPH and secondary PH patients, 11 lesions (7 from the PPH group, 4 from the secondary PH group) were examined for clonality of the hyperplastic smooth muscle cells in hypertensive, muscular pulmonary arteries with marked medial hypertrophy. PCR amplification was successful in all 11 vessels. Only one vessel sampled (from a patient with PPH) had unbal-

anced methylation, and the clonality ratio of the 11 lesions was 0.65 ± 0.30 (range 0–0.94) (Figs. 1–5).

Discussion

We demonstrated that the endothelial cell proliferation in the majority of the PPH plexiform lesions examined occurs indeed via a monoclonal expansion, whereas in secondary PH (due to high pulmonary blood flow or associated with the CREST syndrome), plexiform lesions contain a polyclonal population of endothelial cells. These findings indicate that the endothelial cell proliferation in PPH is of fundamental importance, with major implications regarding the pathogenesis and diagnosis of PPH. This is the first demonstration of monoclonality of endothelial cells in a nonneoplastic vascular disease (13).

Our data showing a monoclonal endothelial cell proliferation in PPH suggests that a somatic genetic alteration, associated or not to a unique genetic susceptibility, may represent the underlying factor that allows a clonal expansion of pulmonary endothelial cells. A monoclonal cellular expansion occurs when a single cell, affected by a rare and random initiating event (such as a somatic mutation), acquires an important growth advantage over the normal cells. This process of selective clonal cell outgrowth represents the early stage of neoplasia as demonstrated in the sequence and progression from colonic gland hyperplasia to adenoma and colon cancer (11). Clonal expansion is also present in other types of cancers (19–23), and monoclonality has been used to confirm the neoplastic nature of several mesenchymal tumors, including desmoid tumors (24) and leiomyomas (25). Recently, monoclonality of Kaposi's sarcoma, an aggressive and multicentric endothelial cell tumor that occurs in immunosuppressed patients, was demonstrated based on the pattern of X-chromosome inactivation. In Kaposi's sarcoma, the multiple vascular lesions in a given patient showed a uniform pattern of X-chromosome inactivation, suggesting a disseminated monoclonal cancer arising from a circulating stem cell (12). In contrast, the plexiform lesions of a given female PPH patient appear to have independent multifocal clonal origins, since a discordant pattern of methylation of the X-linked human androgen receptor alleles was detected in all but one of the PPH patients.

The main caveat in the interpretation of the data of monoclonality of proliferated endothelial cells in plexiform lesions in PPH is the possible presence of a normal endothelial patch, which has a uniform pattern of X-chromosome inactivation within the affected pulmonary vessel. If the normal patch size is large enough, sampling from randomly reactive endothelial cells within the patch would then result in monoclonality (13). Early studies showed the existence of large patches with a uniform pattern of X-chromosome inactivation of normal cells in several tissues (26, 27), including smooth muscle in the media of the aorta and coronary arteries (13). However, regarding the vascular endothelium, no data on the normal patch size are available. We dealt with this shortcoming by evaluating the clonality of proliferated endothelial cells of plexiform lesions of similar size (similar length and diameter) in both PPH and secondary PH. In addition, we evaluated the clonality of adjacent hyperplastic smooth muscle cells of similar size pulmonary arteries with medial hypertrophy. The mean clonality ratio found in endothelial cell proliferation in secondary PH was 0.68, smaller than in lung parenchyma samples ($P < 0.01$, Student's *t* test). Similar findings were obtained when we analyzed
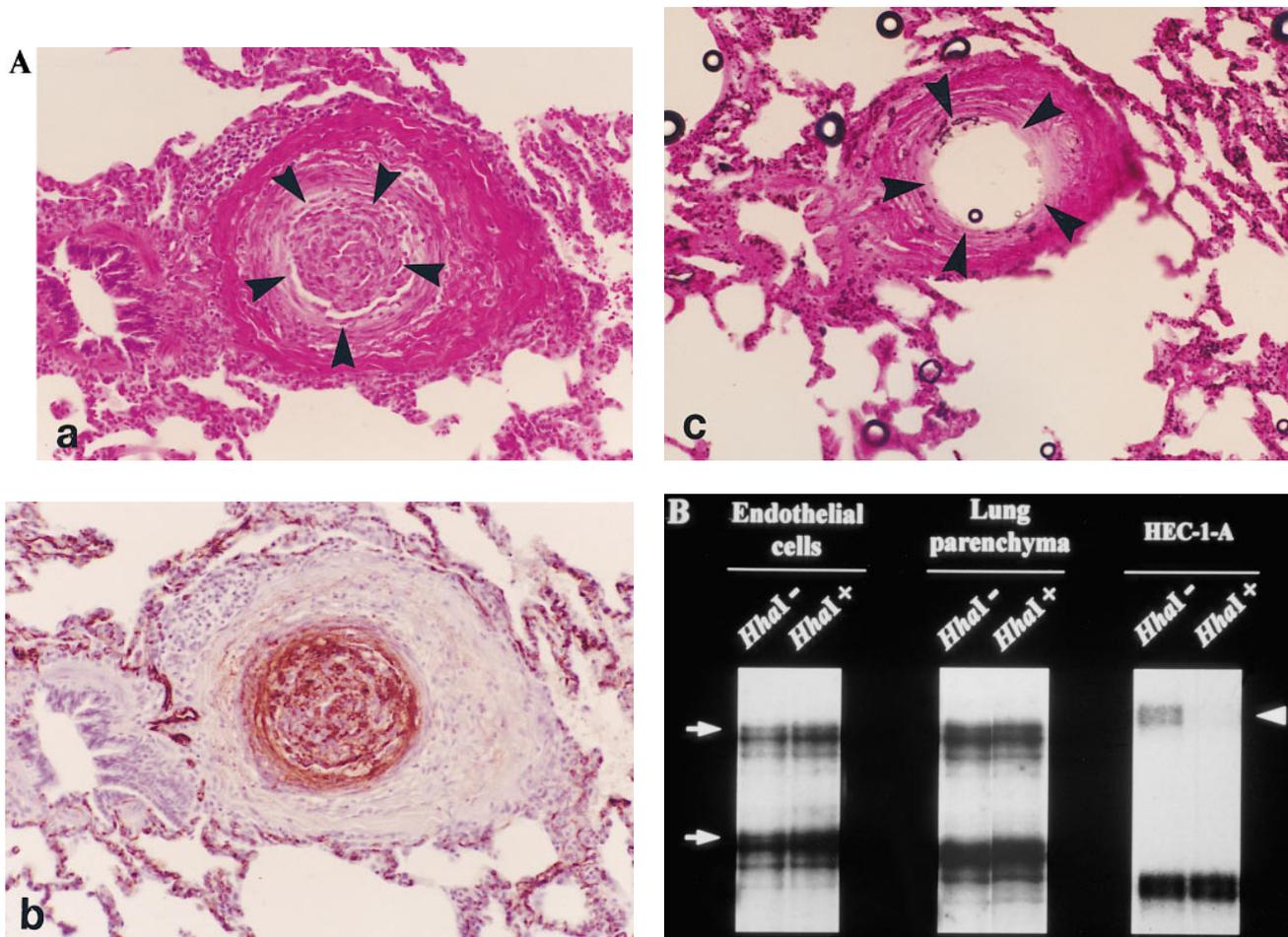
Figure 3. Assessment of clonality of endothelial cells in a plexiform lesion in secondary PH (patient 9). (*A*) Morphology of a plexiform lesion in secondary PH showing (*a*) prominent intravascular proliferating endothelial cells (hematoxylin and eosin staining, ×200) (*arrowheads*), (*b*) intense immunostaining for Factor VIII related antigen (immunoperoxidase ×200), and (*c*) that the proliferating endothelial cells were harvested selectively by microdissection for subsequent PCR amplification (eosin staining, ×200) (*arrowheads*). (*B*) Clonality analysis of endothelial cells in a plexiform lesion (shown in *A*) in secondary PH. In proliferated endothelial cells of a plexiform lesion, two parental alleles (*arrows*) are present after HhaI digestion (lane *HhaI+*) (clonality ratio = 0.95, refer to Methods), representative of balanced methylation pattern in a polyclonal population of cells. In the lung parenchyma, both alleles are equally amplified after HhaI digestion, excluding the possibility of unequal lyonization of normal tissue in this patient. We used DNA from the human endometrial adenocarcinoma cell line HEC-1-A as a positive control for PCR and restriction digestion. In HEC-1-A cells, one allele is absent (*arrowhead*) after HhaI digestion (lane *HhaI+*), consistent with the monoclonality of this cancer cell line.

the clonality of smooth muscle cells of small pulmonary arteries with medial hypertrophy, which had a mean clonality ratio of 0.65. The clonality ratio of the lung parenchyma approached 1, possibly because of a more uniform distribution of multiple patches representing the methylation of the maternal or paternal X-chromosome. The monoclonality found in PPH was not due to sampling within a normal endothelial cell patch, since in secondary PH, plexiform lesions of comparable sizes and similar morphology to those sampled in PPH were uniformly polyclonal.

We postulate that the pathogenetic mechanisms involved in the generation of the polyclonal endothelial cell proliferation in pulmonary hypertension are distinct from those causing the monoclonal growth seen in PPH. The polyclonal nature of the endothelial cell proliferation in all of the plexiform lesions in secondary PH suggests that pulmonary endothelial cells may proliferate due to factors such as high shear stress associ-

ated with high pulmonary blood flow or inflammation (as in the patients with PH and CREST syndrome). High shear stress can activate both immediate early gene expression in cultured human endothelial cells (28) and the synthesis of growth factors such as PDGF and TGF-β (29, 30), which may be involved in the growth of pulmonary endothelial cells in secondary PH. It is possible that these mechanisms account for the infrequent occurrence of polyclonality in scattered plexiform lesions in three of the PPH patients.

The multicentric monoclonal expansion of pulmonary endothelial cells in plexiform lesions in PPH resembles that present in the multifocal neoplasia formation in familial adenomatous polyposis (FAP). Novelli et al., in an elegant study of clonality using the Y-chromosome to probe for clonality in an XO/XY FAP patient, demonstrated that microcryptal adenomas are monoclonal and exhibit a concordant Y-chromosome genotype with that of the normal cells at the base of the
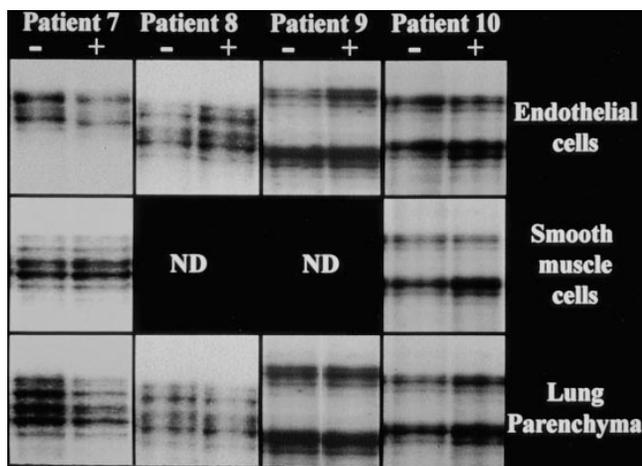
Figure 4. HUMARA of representative examples of polyclonal plexiform lesions, pulmonary artery smooth muscle cells, and lung samples in secondary PH patients 7–10. −, Without HhaI digestion; +, HhaI-digested sample. *ND*, Not done.

Figure 5. Summary of clonality analysis by HUMARA of endothelial cell proliferation in the plexiform lesions and of hyperplastic smooth muscle cells in small pulmonary arteries with medial hypertrophy, in patients with PPH or secondary PH. In four PPH patients, unbalanced methylation, diagnostic of monoclonal composition of the proliferated endothelial cells, was found in 17 (77%) of 22 plexiform lesions. 13 of these 17 lesions showed complete knock-out (clonality ratio = 0) of one allele after restriction digestion with HhaI, as shown in Fig. 1 and Fig. 2 *A*. The pattern of methylation of the human androgen receptor alleles of the proliferated endothelial cells in different plexiform lesions in a given female patient was discordant in three of four patients. Overall, the small allele (*open squares*) was methylated preferentially in 10 lesions, and the larger allele (*filled squares*) predominated in the remaining 7. The polyclonal lesions in PPH patients with a clonality ratio > 0.25 are shown in Fig. 2 *B*. In four secondary PH patients, all 19 lesions showed balanced methylation by HUMARA (clonality ratio > 0.25), diagnostic of polyclonal composition of the proliferated endothelial cells from three patients with congenital heart disease with left to right shunt (*dotted squares*) and from one patient with CREST syndrome (*dotted crosses*). Clonality of hyperplastic smooth muscle cells was analyzed in 11 small pulmonary arteries with medial hypertrophy from PPH (*open circles*) or secondary PH (*open triangles*) patients. Balanced methylation was found in 10 of these lesions (91%).

same colonic crypt (31). These crypt cells act as stem cells giving rise to the cell population in the normal intestinal glandular cells. Because of the findings of monoclonality, expansive growth, and lack of regression of the plexiform lesions in PPH, we hypothesize that as in FAP, the cell proliferation occurs due to the presence of stem cell–like endothelial cells in the pulmonary circulation. These stem-like cells, present in specific sites such as branching points of pulmonary muscular arteries, would have the key properties of clonal growth expansion, self-renewal, and differentiation (32). The particular microenvironment of lung circulation, exposed to extreme variations in oxygen tension and responsible for drug metabolism, may account in part for the unique structural and genetic nature of plexiform lesions in PPH compared with systemic vascular diseases.

The segmental nature of the endothelial growth in plexiform lesions in both primary and secondary PH results in almost complete obliteration of the vascular lumen and irreversible remodeling of the pulmonary vascular wall, as assessed recently by us in three-dimensional reconstruction studies of plexiform lesions (Cool, C.D., J.S. Stewart, P. Werahera, G.J. Miller, R.L. Williams, N.F. Voelkel, and R.M. Tuder, manuscript submitted for publication). We observed that the endothelial cells in these lesions express intensely and uniformly the vascular endothelial growth factor receptor KDR, and segregate phenotypically into the cyclin-kinase inhibitor p27/Kip1− cells in the central core of the lesion and p27/Kip1+ cells in areas with early blood vessel formation. These findings suggest that these lesions are heterogeneous and dynamic, and evolve not only in a spatial distribution sense, but also at the cellular level.

Plexiform lesions can, in principle, occur as monoclonal or polyclonal endothelial cell proliferation. To date, the difference in the clonality status of proliferating endothelial cells of the plexiform lesion is the only finding, irrespective of the clinical history, to discriminate between the vascular lesions of patients with PPH and patients with secondary PH. Our finding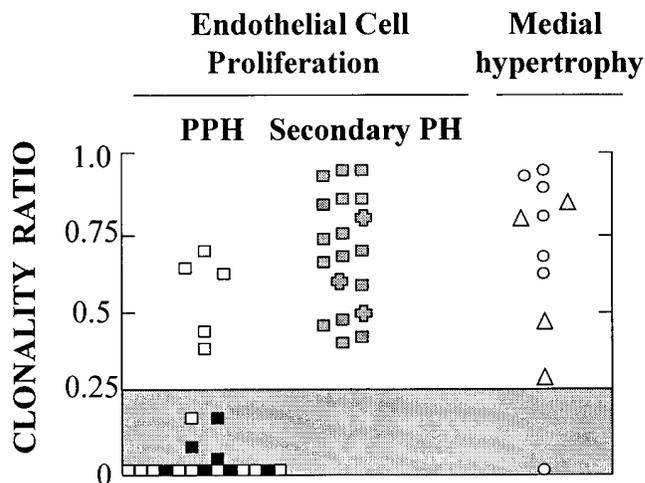s may provide molecular tools to determine whether the pulmonary hypertension, especially in cases associated with anorexigen intake or HIV infection, is primary (monoclonal) or secondary (polyclonal), due to drug or virus infection.

Note added in proof: Three plexiform lesions of an additional PPH patient showed a monoclonal endothelial cell population (clonality ratios of 0, 0.02, and 0.16).

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