

Oral Mucosal Microvascular Abnormalities: An Early Marker of Bronchopulmonary Dysplasia

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ABSTRACT

An abnormal pulmonary vasculature has been reported as an important component of bronchopulmonary dysplasia (BPD). We tested the hypothesis of an early abnormal vascular network pattern in infants with BPD. Fifteen infants with BPD (nine boys and six girls; gestational age 27.5 ± 2.0 wk; birth weight 850 ± 125 g) and 15 sex- and gestational age-matched infants (nine boys and six girls; gestational age 27.6 ± 2.6 wk; birth weight 865 ± 135 g) were examined on postnatal days 1 and 28. BPD infants showed a significantly higher prevalence of histologic chorioamnionitis ($p = 0.009$), as well as higher intubation duration ($p = 0.0004$), oxygen supplementation ($p < 0.0001$), and initial illness severity ($p = 0.0002$) than the BPD-negative population. The lower gingival and vestibular oral mucosa was chosen as the study area. The blood vessel area was determined, and the oral vascular networks were characterized by analyzing their complexity (D , at two scales: $D 1-46$, $D 1-15$), tortuosity

(D_{min}), and randomness (L-Z) of the vascular loops. Infants with BPD showed a significantly lower blood vessel area as well as a higher vascular network complexity ($D 1-46$, $D 1-15$, and L-Z) than control subjects ($p < 0.0001$). Our findings provide a new early clinical sign in BPD and stress the importance of an early disorder in the oral mucosal vascularization process in the disease pathogenesis. (*Pediatr Res* 56: 927-931, 2004)

Abbreviations

BPD, bronchopulmonary dysplasia
BVA, blood vessel area
D, fractal dimension
 D_{min} , minimum path fractal dimension
HCA, histologic chorioamnionitis
L-Z, relative Lempel-Ziv complexity

Bronchopulmonary dysplasia (BPD) remains an important cause of mortality and morbidity in preterm infants. BPD is infrequent in infants with birth weight >1200 g or with gestation >30 wk, whereas among infants with birth weight <1000 g, a prevalence of $\sim 30\%$ has been reported (1,2). To date, the pathogenesis of BPD remains unclear. Besides the well-known oxygen toxicity, volutrauma, and mechanical injury mechanisms (3), antenatal or postnatal inflammatory response (4,5) and leakage of plasticizers from endotracheal tubes (6,7) have been more recently associated with injury to the immature lung and/or the development of BPD. Delayed

and disturbed formation of the terminal respiratory units have been reported in BPD (8), together with a disordered vascular development and a decreased production of angiogenic factors (9,10). In the past two decades it has been realized that many biologic systems have no characteristic length scale, thus having fractal or self-affine properties (11). Fractals are of great value in biology, where surface phenomena are of crucial importance and a number of complex anatomic structures, including arterial and venous trees, also display fractal-like geometry. We tested the hypothesis of an abnormal mucosa vascularization and network pattern in BPD by using fractal analysis.

METHODS

Fifteen consecutive preterm infants with BPD (nine boys and six girls; gestational age 27.5 ± 2.0 wk; birth weight 850 ± 125 g) were examined, together with 15 sex- and gestational

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age-matched control infants without BPD (nine boys and six girls; gestational age 27.6 ± 2.6 wk; birth weight 865 ± 135 g). A diagnosis of BPD was made on the basis of all of the following criteria (12): 1) requirement of intermittent positive-pressure ventilation ≥ 3 d during the first week after birth, 2) clinical signs of chronic respiratory disease persistent for >28 d, 3) requirement of supplemental oxygen for >28 d to maintain an arterial partial pressure of oxygen >50 mm Hg, and 4) typical changes on the chest radiograph. All of the participating infants were evaluated during the first day of life and at 28 d. Initial severity of illness was assessed using the five-item scoring system Clinical Risk Index for Babies II (CRIB-II) (13). Accordingly, all infants were examined after informed parental consent, and the ethics committee approved the study.

Placental histology. Placental histologic examination was performed as previously described (14), and a diagnosis of histologic chorioamnionitis (HCA) was made given the presence of at least 10 polymorphonuclear leukocytes per field in 10 nonadjacent 400-power fields in membranes and/or placental chorionic plate. Pathologists were unaware of the neonatal outcomes (interobserver agreement in identifying histologic CA: $\kappa = 0.97$, SE = 0.015, 95% CI 0.93–0.98).

Oral mucosal vascular analysis. The lower gingival and vestibular oral mucosa was chosen as the study area, because of its accessibility. High-resolution photographs of the selected areas were acquired using a Yashica Dental Eye photcamera with an automated on-axis flashbulb and a 55-mm, f 1:4 Yashica lens. Kodak Elite Chrome 100 ISO/21 DIN films were used and developed according to the standard E-6 procedure (Fig. 1). Images were digitized using a Canon Canoscan FS2710 scanner (color resolution 680 dpi, $\times 6.67$ magnifica-

tion) with a Windows 98 operating system. The percentage blood vessel area (BVA%) was determined by calculating the percentage area of the imaged oral mucosa covered by vascularization, using the Image Pro-Plus version 1.3 image analysis software (Image Pro-Plus-Media Cybernetics, Silver Spring, MD), with mean intra- and interobserver variability (coefficient of variation) of $2.5 \pm 1.8\%$ and $5.6 \pm 3.2\%$, respectively ($n = 6$; Fig. 2). The obtained vascular network images were converted into binary skeletonized form for geometric pattern analysis. Manual outline of the two-dimensional (2-D) vascular network's trajectories was performed using Adobe Photoshop (Adobe Systems, San Jose, CA) on a Sony 19-inch Trinitron Multiscan G420 screen ($16 \mu\text{m}/\text{pixel}$ resolution) by two operators who were unaware of the patients' study population. Nonreadable areas were $<5\%$ of the total area. Images were processed to threshold the vessel network without background interference, and the networks were subsequently converted into an outline of single pixels (Fig. 3). The local fractal dimension (D) was determined for two regions of box lengths $<740 \mu\text{m}$ [pixels 1–46, D^{1-46}] and $<140 \mu\text{m}$ [pixels 1–15, D^{1-15}], respectively. The fractal dimensions of 2-D skeletonized images were measured using the box-counting algorithm, according to the relation $N(L) \sim L^{-D}$, where L is the box size, and $N(L)$ is the number of squares (15). The measuring procedure was calibrated against shapes of known fractal dimension having an inaccuracy of $\pm 2\%$.

Although the scaling plots (number of boxes versus box size, log-log plots) for the examined microvascular networks at two different scales [D^{1-15} , D^{1-46}] indicated a crossover between a uniform planar pattern ($D = 2$, approximate slope for small box sizes) and lines ($D = 1$, approximate slope for large box sizes), thus suggesting that the networks are not properly fractal (data not shown), the box-counting method was maintained as a means to characterize the pattern geometry.

The fractal dimension of the minimum path, D_{min} , was computed for each vascular cluster from the power law $l_c = r^{D_{min}}$, where D_{min} is the exponent that governs the dependence of the minimum path length between two points (l_c) on the Pythagorean distance (r) between them in a fractal random material (16). After enlarging the image to $\times 2.4$ magnification, thinning to one pixel, and discarding all areas with a diameter less than pixels, the half perimeter (x_i) and the maximum diameter (y_i) of either the vessel loops or vessel-free areas in the single-pixel 2-D pattern were measured using an automated

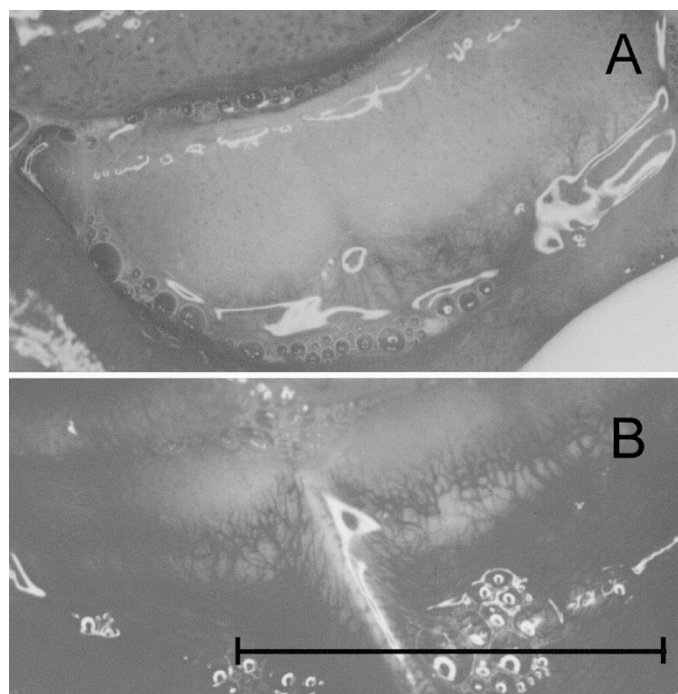


Figure 1. Original photographs of lower vestibular oral mucosa in a typical infant with BPD (A) and in a gestational age-matched control neonate (B). The size bar corresponds to 1 cm.

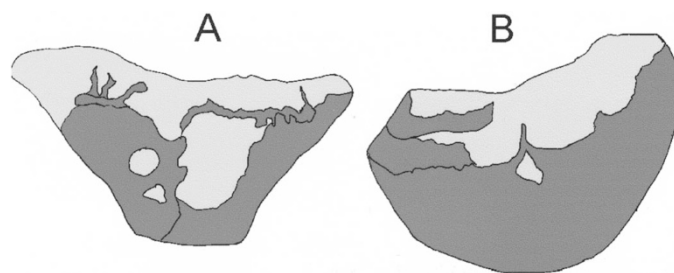


Figure 2. Schematic representation of the BVA% as determined by calculating the percentage area of the imaged oral mucosa covered by vascularization. (A) BPD infant. (B) Control newborn. Red, vascularized areas; grey, avascular areas.

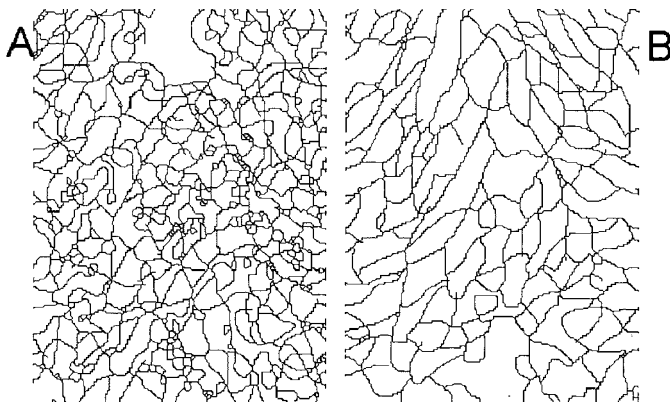


Figure 3. 2-D skeletonized microvascular networks in an infant with BPD (A) compared with a gestational age-matched control subject (B).

procedure (Image Pro-Plus version 1.3 image analysis software; examined areas for each sample: 500–1000). The slope of the Log/Log plot x_i/y_i represented D_{min} . The method was validated with the original one by Herrmann and Stanley (16) with a maximum shift of $\pm 3\%$. To determine the algorithmic complexity of the vascular patterns, we calculated relative Lempel-Ziv (L-Z) (17) values according to the Kaspar and Schuster algorithm (18) using the Chaos Data Analyzer version 2.1 software package (CDA: Pro, The Academic Software Library, North Carolina State University, Raleigh, NC). It was accepted that relative L-Z values could range from near 0 for a deterministic equation to ~ 1.0 for destructured random phenomena (*i.e.* sinus function L-Z 0.0044; white noise 1.047). The vascular network analysis was determined to be reproducible, given mean intra- and interobserver coefficients of variation of ≤ 5.0 and $\leq 10\%$, respectively.

Statistics. Variables were tested for normality using either the χ^2 or Kolmogorov-Smirnov tests. Group sizes were calculated to give a 90% power to detect differences between group means of 1 SD of each normally distributed variable, with an α value of 0.05. Differences among group means were analyzed by either the *t* test, with 95% confidence intervals of differences of means between the groups, or the Wilcoxon's test, as appropriate. Associations between normally distributed, continuous variables were tested using univariate regression analysis. A two-sided $p < 0.05$ was considered to indicate statistical significance, and the Bonferroni corrected significance ($p < 0.05/n$) levels were used for multiple *t* tests. The

MedCalc version 7.2.1.0 statistical software package (MedCalc Software, Mariakerke, Belgium) was used.

RESULTS

BPD infants showed a significantly higher prevalence of HCA ($p = 0.009$) than control infants (Table 1). BPD infants also showed a significantly higher intubation ($p = 0.0004$) and O_2 supplementation duration ($p < 0.0001$), as well as higher Clinical Risk Index for Babies II scores ($p = 0.0002$) than the BPD-negative population. In contrast, the differences in proportion of endotracheal intubation ($p = 0.084$), arterial partial pressure of carbon dioxide/fraction of inspired oxygen ratio ($p = 0.34$), and intubation duration/supplementary O_2 duration ($p = 0.33$) were not statistically significant.

BPD infants on day 1 showed significantly lower BVA% than control subjects (35.05 ± 8.86 versus $65.31 \pm 12.43\%$; $p < 0.0001$), and their oral vascular networks showed significantly higher complexity [D^{1-46} 1.92 ± 0.036 versus 1.75 ± 0.091 ($p < 0.0001$); D^{1-15} 1.29 ± 0.072 versus 1.17 ± 0.032 ($p < 0.0001$); L-Z 0.85 ± 0.036 versus 0.71 ± 0.084 ($p < 0.0001$)] than control subjects (Table 2). An inverse correlation between BVA% and fractal dimension was observed (BVA% versus D^{1-46} : $r = -0.60$, $p = 0.01$; BVA% versus D^{1-15} : $r = -0.61$, $p = 0.0088$). Applying the receiver operating characteristic statistics, a BVA% $\leq 49.17\%$ resulted in correctly classifying the subjects who belonged to the two groups (BPD patients versus control subjects) with 100% sensitivity and 100% specificity. Statistical differences observed on day 1 were maintained on day 28 (Table 3). Nine (60%) of the BPD patients were still on oxygen supplementation at 36 wk postconceptional age.

A pilot study on oral vascular variables in HCA infants without BPD ($n = 20$; 11 boys and nine girls; gestational age 27.6 ± 2.2 ; birth weight 845 ± 130 g) showed results comparable to HCA-negative infants ($n = 20$; 10 boys and 10 girls; gestational age 27.8 ± 2.1 ; birth weight 850 ± 140 g) without BPD [BVA%: 60.7 ± 8.6 versus 67.61 ± 14.08 ($p = 0.12$); D^{1-46} : 1.76 ± 0.072 versus 1.74 ± 0.10 ($p = 0.67$); D^{1-15} : 1.17 ± 0.035 versus 1.18 ± 0.031 ($p = 0.73$); D_{min} : 0.99 ± 0.055 versus 1.00 ± 0.026 ($p = 0.80$); L-Z: 0.74 ± 0.063 versus 0.70 ± 0.094 ($p = 0.13$)].

Table 1. Histologic chorioamnionitis, assisted ventilation, and initial illness severity in extremely low birth weight infants with and without BPD-28 days

Variables	BPD+ ($n = 15$)	BPD- ($n = 15$)
Antenatal steroids	14 (93.3%)	14 (93.3%)
Endotracheal intubation	14 (93.3%)	9 (60%)
Intubation duration (h)	456 (356–745)	24 (9–41)†
Supplementary O_2 duration (h)	1210 (903–1402)	48 (24–72)†
Intubation time/ O_2 time ratio	0.41 (0.29–0.58)	0.51 (0.24–0.62)
PaO_2/FiO_2 ratio	1.30 ± 0.78	1.74 ± 1.57
Histologic chorioamnionitis	13 (86.7%)	5 (33.3%)*
CRIB II	12.71 ± 2.85	7.8 ± 3.42 †

PaO_2 , arterial partial pressure of oxygen; FiO_2 , fraction of inspired oxygen; CRIB II, Clinical Risk Index for Babies II.

* $p < 0.01$.

† $p < 0.0001$.

Table 2. Comparisons of oral vascular mucosal variables at day 1

Variable	BPD (n = 15)	Controls (n = 15)	95% CI of Difference	p value
BVA %	35.05 (8.86)	65.31 (12.43)	22.19 to 38.33	<0.0001
D ¹⁻⁴⁶	1.92 (0.036)	1.75 (0.091)	0.12 to 0.22	<0.0001
D ¹⁻¹⁵	1.29 (0.072)	1.17 (0.032)	0.078 to 0.16	<0.0001
D _{min}	1.02 (0.038)	1.00 (0.035)	-0.012 to 0.042	0.26
L-Z	0.85 (0.036)	0.71 (0.084)	0.089 to 0.18	<0.0001

Values are expressed as means (SD) and confidence interval (CI) of the differences between the two study groups.

Table 3. Comparisons of oral vascular mucosal variables at day 28

Variable	BPD (n = 15)	Controls (n = 15)	95% CI of Difference	p value
BVA %	41.45 (5.78)	66.28 (16.47)	15.49 to 33.95	<0.0001
D ¹⁻⁴⁶	1.90 (0.017)	1.76 (0.058)	0.11 to 0.18	<0.0001
D ¹⁻¹⁵	1.26 (0.075)	1.17 (0.037)	0.049 to 0.14	<0.002
D _{min}	1.03 (0.048)	1.01 (0.037)	-0.0051 to 0.059	0.096
L-Z	0.85 (0.019)	0.69 (0.10)	0.11 to 0.22	<0.0001

Values are expressed as means (SD) and CI of the differences between the two study groups.

DISCUSSION

The main findings of the present study indicate the presence of a previously unrecognized early disorder of reduced oral mucosal vascularization process in infants who subsequently develop BPD. The lower gingival and vestibular oral mucosa proved a readily accessible site for analysis of BVA%. The findings of a significantly lower BVA% by the first postnatal day support the hypothesis of a key role for early vascular abnormalities in the pathogenesis of BPD and seem to confirm that BPD may begin in intrauterine life (4,5). We have found that these abnormalities are not limited to the lung, but the BPD-associated oral microvessel networks exhibit a significantly increased overall complexity at both larger and smaller scales, together with an increased destructured randomness. The D¹⁻⁴⁶ results for the vascular networks of the BPD infants display intermediate characteristics between the diffusion-limited aggregation behavior of control networks and the percolation-like scaling of tumor-associated vascular networks (19). In contrast, vessel tortuosity was comparable between the groups. Although the mechanisms underlying this clinical observation remain to be clarified, angiostatic agents (9,20) may play a previously unrecognized, major role in producing injury to the developing lung. Moreover, an abnormal pulmonary vasculature has been reported as an important component of BPD (9,10). The absence of microvascular changes in HCA patients who subsequently will not develop BPD confirms the multifactorial pathogenesis of the disease where other factors may also play a role, including mechanical trauma and oxygen toxicity (3-5), pulmonary edema associated with patent ductus arteriosus and/or excess fluid administration (21), nutritional deficiencies (22), a predisposition to airway reactivity (23), early adrenal insufficiency (24), and di-(2-ethylhexyl)-phthalate release from endotracheal tubes (6,7). Recent preliminary research indicates a reduced expression of vascular endothelial growth factor receptor 1 (25) in fetal capillaries in preterm placentas with chorioamnionitis, which is in line with

the findings of altered vascularization in infants who have HCA and subsequently develop BPD. The correlation between antenatal infection/inflammation (*i.e.* HCA) and BPD/chronic lung disease has been well documented (26,27). However, a significant interaction between chorioamnionitis and mechanical ventilation and/or postnatal sepsis in the pathogenesis of chronic lung disease has been reported (28), and the importance of ventilation strategy on the pulmonary outcome of premature infants has been stressed (29).

Molecular biology offers ever-increasing spectacular successes, although a detailed inventory of genes, proteins, and metabolites is not itself sufficient to understand biologic complexity (30). Elucidating the fractal and nonlinear mechanisms involved in physiologic control and complex signaling networks is emerging as a major challenge in the postgenomic era (31). In particular, for medical images, fractals geometry is increasingly used as an index of irregularity, thus evaluating angiogenic processes (32). To deliver oxygen and nutrients to all of the cells of the body, blood vessels must have fractal properties (33), and their branching pattern is very similar to the growth model called diffusion-limited aggregation. Furthermore, it has previously been shown that fractal dimension (*D*) and other fractal characteristics can be used to identify abnormal vascular patterns, including corneal (34), retinal (35), and tumor-associated neovascular growth (19,36). The precise mechanisms underlying the observed increased vascular complexity remain unclear, although extracellular matrix is known to play an important role in angiogenesis and blood vessel geometry (37,38), and changes in extracellular matrix components have been previously reported in experimental models and patients with BPD (39,40). Although study of larger populations should be performed to confirm our preliminary observations, the findings of early changes in the oral mucosal vascularization associated with BPD may be crucial in selecting candidate patients for treatment as specific treatments become available regarding vasculogenesis.

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