Gelatinase activities in the airways of premature infants and development of bronchopulmonary dysplasia

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Mechanical ventilation, oxygen therapy, and airway inflammatory responses have all been implicated in the development of bronchopulmonary dysplasia (BPD). We, therefore, evaluated correlations between gelatinase activities (metalloproteinase; lung development; newborn; extracellular matrix)

PREMATURE NEONATES WITH HYALINE membrane disease are at risk of developing bronchopulmonary dysplasia (BPD), as a sequel of both the disease and its treatment. The pathogenesis is unclear, but BPD is thought to result from damage to an immature lung. Mechanical ventilation, oxygen therapy, and airway inflammatory responses have all been implicated in the development of BPD (19). These insults appear to interfere with normal lung maturation, which is incomplete at birth. In particular, they appear to alter alveolar formation, on the basis of morphometric studies of severe BPD (3, 24). Alveolar formation is characterized by the multiplication of alveolar septa and the thinning of interalveolar walls, both of which require intense remodeling of the pulmonary extracellular matrix (4). Changes in matrix turnover have been experimentally linked to abnormal alveolar formation (20). The proteinase-antiproteinase balance in the lung is a key factor in harmonious matrix turnover. In particular, matrix metalloproteinases (MMPs), which can synergistically digest the major macromolecules of connective tissue matrices, have been implicated in physiological regulation of lung growth. The MMP gelatinase A (MMP-2) has been shown to play a role in the major collagen turnover and that inadequate MMP-2 levels in premature lungs exposed to insults could contribute to impaired lung growth and thus to BPD. We also postulated that MMPs may be secreted in excess during the inflammatory response associated with hyaline membrane disease and may, therefore, lead to tissue degradation. Gelatinase B (MMP-9) is the main MMP released by inflammatory cells such as neutrophils and alveolar macrophages (17, 35) and has been implicated in human lung diseases such as cystic fibrosis (11) and asthma (22). We thus studied premature infants with hyaline membrane disease to determine whether the profile of gelatinase activities at birth was associated with the subsequent risk of BPD and to examine the possible link between the early postnatal time course of gelatinase activities and clinical outcome. We also tested airway secretions for tissue inhibitor of metalloproteinases (TIMP)-1, a key specific inhibitor of MMPs that forms tight complexes with activated MMP-2 and MMP-9 (26, 36).
PATIENTS AND METHODS

Reagents. All reagents were obtained from Sigma Chemical (St. Louis, MO), unless otherwise specified.

Study design. We measured MMP-2, MMP-9, and TIMP-1 levels in airway secretions of highly premature neonates with recent-onset hyaline membrane disease and tested their correlation with clinical outcome. Tracheal aspirates were obtained from intubated premature neonates within 6 h of birth (day 0), before surfactant instillation. This first sample was considered to reflect physiological metalloproteinase activity. Infants still intubated on days 3 and 5 were resampled. Changes in MMP-2, MMP-9, or TIMP-1 levels between day 0 and day 3 or 5 were considered to reflect the airway response to insults. This study was part of a clinical research program (Programme Hospitalier de Recherche Clinique) approved by the local ethics committee, and the parents’ informed consent was obtained.

Study group. Sixty-four neonates with gestational age ≤30 wk were included in the study from February 1997 to February 1999. They all had clinical and radiological diagnostic criteria of hyaline membrane disease. Infants suspected to be born after chorioamnionitis or having meconium infection were excluded from the study. The mean term was 27.8 ± 0.2 (SE) wk (range 24–30 wk), and mean birth weight was 994 ± 28 g (600–1,540 g). Only one of the neonates had intrauterine growth retardation. Corticosteroids had been administered to the mother in 33 cases. Forty-five neonates required prolonged mechanical ventilation and were resampled on day 3 or 5.

Peak inspiratory pressure (PIP) and the fraction of inspired oxygen (FIO2) were noted just before tracheal aspiration. The diagnosis of BPD was based on the continued need for oxygen supplementation at a postconceptional age (PCA) of 36 wk (31).

Collection of tracheal secretions. Gentle tracheal aspiration is routinely performed in our unit to maintain airway patency in intubated neonates. These aspirates were the samples used for this study. Suction was preceded by instillation of 0.2 ml of isotonic saline. After three to five ventilator breaths, suctioning was performed through a small catheter, and the aspirated material was collected in an infant mucous extractor (VYGON, Ecouen, France). It was then diluted in 0.2 ml isotonic saline, gently vortexed, and centrifuged at 1,200 rpm and 4°C for 10 min. The supernatant was recovered (mean volume: 190 ± 12 μl) and stored at −80°C. The cell pellet was resuspended in 0.2 ml of 0.1% dithiothreitol. Cell smears were stained with the standard May-Grünwald-Giemsa procedure, and differential cell counts were done. This technique was very close to others previously described (15, 32). Although some authors used higher aliquot volumes and higher aliquot numbers, those studies were concerned with neonates with higher gestational age and higher birth weight than our population (9, 16). In our experience, higher aliquot volumes were badly tolerated in numerous premature infants weighing <1,000 g. Because of the small volume of saline instilled into the airways, the dilution factor induced by our technique was thought to be low and was evaluated in preliminary experiments by using secretory IgA (sIgA) as a dilution marker (Immun Diagnostik, Bensheim, Germany). The intersubject coefficient of variation of sIgA in 60 samples was 18.2%, demonstrating a much lower variability in the dilution factor than that observed with higher aliquot volumes (9). Furthermore, our laboratory previously demonstrated with this method a high correlation between expression of results per milliliter of supernatant and per nanogram of sIgA (r = 0.823; P < 0.0001) (8). Thus the dilution factor induced by our method is low and does not introduce significant bias in results expressed per milliliter of supernatant. Given the fact that the small volume of sample recovered after aspiration does not allow systematic measurement of sIgA in addition to other parameters, results were expressed per milliliter of supernatant.

Zymographic gelatinase analysis. Supernatants of tracheal aspirates were analyzed by electrophoresis in 11% (wt/vol) polyacrylamide gels containing 1 mg/ml gelatin in the presence of SDS in nonreducing conditions. After electrophoresis, gels were washed in 2.5% Triton X-100 for 1 h and then rinsed briefly and incubated at 37°C for 48 h in buffer containing 100 mM Tris-HCl, pH 7.40, and 10 mM CaCl2. The gels were then stained with Coomassie brilliant blue R250 and restained in a solution of 7.5% acetic acid and 5% methanol. Zones of enzyme activity were indicated by negative staining; areas of proteolysis were seen as clear bands against a blue background. The activities were attributed to metalloproteinases after determining inhibition profiles. Gels were incubated in Tris buffer containing one of the following metal chelators: 5 mM EDTA (10 μl), the cysteine protease inhibitor N-ethylmaleimide (2 mM), or the serine protease inhibitor phenylmethylsulfonyl fluoride (2 mM).

Enzyme activities in the gel slabs were quantified by means of image analysis (NIH Image software 1.52 for Macintosh), on the basis of both the surface area and the intensity of lysis bands. Results were expressed in arbitrary units (AU) as AU per 48 h per microliter of supernatant. To verify that the method was linear over the range of activities measured in unknown samples, we evaluated activities in increasing volumes of a given aliquot and found that AU values correlated linearly with the sample volume (r2 = 0.99). Repeated evaluation of the same sample showed intragel and intergel coefficients of variation <10%. Total MMP-2 level was calculated as the sum of activities measured at 72 kDa (proenzyme) and 68 kDa (activated enzyme). Similarly, total MMP-9 level was calculated as the sum of activities measured at 92 kDa (proenzyme) and 88 kDa (activated enzyme). The fraction of activated gelatinase was calculated as follows: activated/(activated + proenzyme).

Immunoblotting. The 72- and 92-kDa gelatinase activities were characterized by immunoblotting. Supernatants of tracheal aspirates from five infants were pooled and partially purified by using gelatin-Sepharose chromatography, as previously described (11). Samples were separated by SDS-PAGE and were electrophoretically transferred to nitrocellulose. After saturation of excess protein binding sites with 5% cow’s milk in 0.05 M Tris-HCl and 0.15 M NaCl, pH 7.6 (Tris-buffered saline) for 1 h at room temperature, the nitrocellulose was incubated with a specific rabbit antibody to human 92-kDa MMP-2t 1:1,000 dilution (Clinisciences, Montreouge, France) or to human 72-kDa MMP-2t 1:5,000 dilution (Chemicon, Temecula, CA) in the above buffer for 1 h at room temperature. After thorough washing with Tris-buffered saline, the samples were incubated with a peroxidase-labeled swine anti-rabbit antibody (1:1,000 dilution) in Tris-buffered saline containing 5% cow’s milk for 1 h at room temperature. After washing, the immunoblots were visualized by using an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK).

Free gelatinolytic activity. Gelatinolytic activity was assayed by using [3H]gelatin with a specific activity of 37 kBq/100 μg. [3H]gelatin (50 μg) was incubated with the sample at 37°C for 48 h in a reaction mixture containing 50 mM Tris-HCl, 5 mM CaCl2, and 0.02% NaN3, pH 7.40 (final volume 500 μl). At the end of the assay, samples were cooled.
RESULTS

Gelatinase activities and TIMP-1 levels in airway secretions at birth. Sixty-four children were sampled within 6 h of birth. Nine of these neonates died before reaching a PCA of 36 wk. Eighteen of the 55 survivors still needed oxygen supplementation at 36 wk PCA and were thus considered to have BPD.

Various zymographic profiles of total gelatinase activity were observed according to the degree of prematurity and postnatal age (Fig. 1). These activities were identified as metalloproteinases, as they were completely inhibited by 10 mM EDTA but not by 2 mM phenylmethylsulfonyl fluoride or 2 mM N-ethylmaleimide. Immunoblotting with anti-human MMP-9 and anti-human MMP-2 antibodies identified the relevant metalloproteinases (Fig. 1). MMP-2 was the predominant form on day 0, whereas MMP-9 levels were low. Median values are reported in Table 1. Activated MMP-2 (68-kDa band) was observed in some neonates, contrary to activated MMP-9 (88-kDa band). Very low levels of free gelatinolytic activity were found in most neonates.

Both birth weight and gestational age significantly influenced values on day 0, but multivariate regression analysis showed that birth weight was the main factor. Birth weight significantly influenced total MMP-9 level and the fraction of activated MMP-2 but not total MMP-2 level or the TIMP-1 level. The neonates with the lowest birth weights were characterized by very low MMP-9 level on day 0 (r = 0.42; P = 0.0005) and by a small fraction of activated MMP-2 (r = 0.40; P = 0.001).

Median MMP-9 level (25th to 75th percentile) was significantly lower in neonates exposed to antenatal corticosteroids than in those unexposed: 0.4 (0.2–1.0) vs. 1.0 (0.6–1.6) × 10^5 AU·µl⁻¹·h⁻¹, respectively (P < 0.05). MMP-2 levels were not significantly influenced by steroid exposure: 1.6 (0.6–2.8) vs. 1.6 (1.1–2.7) × 10^5 AU·µl⁻¹·h⁻¹, respectively.

On day 0, logistic regression showed that total MMP-2 level, FIO₂, and birth weight, but not MMP-9 or TIMP-1 levels, were markers significantly associated with BPD outcome. Total MMP-2 level on day 0 was the strongest marker; neonates who developed BPD had a significantly lower MMP-2 level than other neonates (P = 0.0004, Fig. 2). A receiver-operator characteristic curve was plotted to determine the sensitivity and specificity of possible cutoff points for the MMP-2 level in discriminating between infants without BPD and those with BPD (6). The receiver-operator characteristic curves plot all possible combinations between the true positive ratio (sensitivity; y-axis) and the false positive ratio (1 − specificity; x-axis) as one varies the definition of positivity. Different points were, therefore, obtained by varying MMP-2 levels taken as the criterion for positivity. The best cutoff associated with the subsequent development of BPD, defined as the point of the curve closest to the upper left-hand corner,
was an MMP-2 level of $\leq 0.8 \times 10^5$ AU$\cdot$µl$^{-1}$·48 h$^{-1}$ at birth. This cutoff yielded a sensitivity of 61%, a specificity of 92%, a positive predictive value of 79%, and a negative predictive value of 83%. Relative to neonates who remained free of BPD, those who developed BPD also had significantly higher FIO$_2$ values on day 0 ($0.63 \pm 0.05$ vs. $0.43 \pm 0.04$, respectively, $P < 0.004$) and significantly lower birth weights ($901 \pm 46$ vs. $1,055 \pm 38$ g, respectively; $P < 0.02$).

Time course of gelatinase activities and TIMP-1 levels. Forty-five neonates were resampled on day 3, and 36 were sampled a third time on day 5 (Table 2). They did not differ significantly on day 0 from the 19 neonates who were not resampled, except for FIO$_2$ (0.54 ± 0.03 vs. 0.41 ± 0.05, respectively; $P < 0.05$). Fourteen of these 45 neonates still required oxygen supplementation at 36 wk PCA.

Changes in measured parameters for these 45 neonates are summarized in Table 2. Mean total MMP-2 and MMP-9 activities changed significantly with time, in opposite directions. The MMP-2 level fell ($P < 0.0001$), whereas the MMP-9 level increased ($P < 0.0001$). TIMP-1 also increased significantly over time ($P = 0.0003$). Free gelatinolytic activity remained low at all of the time points studied and did not change significantly.

Absolute changes from day 0 to day 3 or 5 in MMP-2 and MMP-9 activities as well as in TIMP-1 levels did not differ between neonates with or without subsequent BPD (Fig. 3). In particular, MMP-9 level increased to the same extent in neonates who developed BPD and in those who did not. Activated MMP-9 (88 kDa) was observed in some neonates on days 3 and 5. The presence of this active form was not associated with BPD outcome. From day 0 to day 5, the MMP-2 level was consistently lower in neonates who went on to develop BPD (ANOVA repeated measures, $P < 0.03$), but the difference at a given day with other neonates was only significant on day 0 (Table 2). Changes in MMP-2, MMP-9, or TIMP-1 levels were not correlated to PIP or FIO$_2$, on days 3 and 5. Stepwise logistic regression analysis, including all data measured on days 0, 3, and 5, showed that BPD onset was associated with a significantly lower MMP-2 level on day 0, lower birth weight, and higher FIO$_2$ values on day 0.

Inflammatory cells in tracheal aspirates. Differential cell counts were interpretable in 25 neonates. Very few inflammatory cells were observed on day 0 (median: $20 \times 10^3$/ml; range: $10–30 \times 10^3$/ml), and most were macrophages. Except in one child, no neutrophils were found on day 0. The median number of inflammatory cells increased on days 3 and 5 to 100 (45–230) and 75 (50–108) $\times 10^3$/ml, respectively. This increase was essentially due to recruitment of neutrophils, which represented 73.5 (65–79.5) and 52.5 (38–66) % of all inflammatory cells on days 3 and 5, respectively.

The number of inflammatory cells correlated with total MMP-9 level ($P < 0.05$). Higher percentages of neutrophils were also associated with higher total MMP-9 level, but the correlation did not reach significance ($P = 0.06$). No significant relation was found between the inflammatory cell count and total MMP-2 level or TIMP-1 levels.

DISCUSSION

The lungs undergo rapid remodeling during late gestation as a functional respiratory unit is formed. Pre-
Table 2. Parameters evaluated in tracheal aspirates from neonates repeatedly sampled on days 0, 3, and 5 of life

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day 0</th>
<th>Day 3</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2, × 10⁵ AU μl⁻¹·48 h⁻¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>1.9 (0.7–3.5)</td>
<td>0.7 (0.5–1.2)*</td>
<td>1.0 (0.4–1.3)*</td>
</tr>
<tr>
<td>BPD−</td>
<td>2.3 (1.5–4.1)</td>
<td>1.0 (0.5–1.3)*</td>
<td>1.0 (0.4–1.5)*</td>
</tr>
<tr>
<td>BPD+</td>
<td>0.6 (0.4–2.7)†</td>
<td>0.6 (0.5–1.1)</td>
<td>0.5 (0.4–1.2)</td>
</tr>
<tr>
<td>Activated MMP-2, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>0.8 (0.1–13.7)</td>
<td>4.2 (0.2–27.3)</td>
<td>4.5 (0.3–23.0)</td>
</tr>
<tr>
<td>BPD−</td>
<td>1.5 (0.1–13.4)</td>
<td>1.2 (0.2–17.0)</td>
<td>1.9 (0.4–22.7)</td>
</tr>
<tr>
<td>BPD+</td>
<td>0.5 (0.1–15.6)</td>
<td>8.2 (2.3–29.1)</td>
<td>8.1 (1.6–17.8)</td>
</tr>
<tr>
<td>MMP-9, × 10⁵ AU μl⁻¹·48 h⁻¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>0.7 (0.2–1.6)</td>
<td>2.3 (1.4–4.3)*</td>
<td>2.7 (1.9–4.5)*</td>
</tr>
<tr>
<td>BPD−</td>
<td>0.8 (0.3–1.8)</td>
<td>2.9 (1.6–4.6)*</td>
<td>3.0 (2.2–5.0)*</td>
</tr>
<tr>
<td>BPD+</td>
<td>0.4 (0.1–1.0)</td>
<td>1.9 (1.4–3.5)*</td>
<td>2.0 (1.3–3.0)*</td>
</tr>
<tr>
<td>Activated MMP-9, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>0</td>
<td>0 (0–13.8)*</td>
<td>0 (0–6.6)*</td>
</tr>
<tr>
<td>BPD−</td>
<td>0</td>
<td>0 (0–13.9)*</td>
<td>0 (0–8.9)*</td>
</tr>
<tr>
<td>BPD+</td>
<td>0</td>
<td>0 (0–12.0)*</td>
<td>0 (0–6)*</td>
</tr>
<tr>
<td>TIMP-1, ng/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>155 (62–316)</td>
<td>395 (131–753)*</td>
<td>387 (177–662)*</td>
</tr>
<tr>
<td>BPD−</td>
<td>167 (79–333)</td>
<td>341 (143–796)*</td>
<td>391 (232–647)*</td>
</tr>
<tr>
<td>BPD+</td>
<td>124 (44–330)</td>
<td>461 (356–736)*</td>
<td>302 (87–639)*</td>
</tr>
<tr>
<td>Free gelatinolytic activity, ng μl⁻¹·48 h⁻¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>10 (8–14)</td>
<td>5 (1–12)</td>
<td>8 (5–21)</td>
</tr>
<tr>
<td>BPD−</td>
<td>12 (9–14)</td>
<td>5 (1–11)</td>
<td>7 (2–17)</td>
</tr>
<tr>
<td>BPD+</td>
<td>9 (8–12)</td>
<td>11 (2–15)</td>
<td>16 (8–22)</td>
</tr>
</tbody>
</table>

*Values are medians with 25th–75th percentiles in parentheses; n, no. of neonates. The fraction of activated gelatinase is calculated as the ratio of the active form to the sum of the active and proforms. Significant difference † compared with day 0 and * compared with BPD−, P < 0.05.

Mature birth may interfere with harmonious extracellular matrix turnover and is, therefore, associated with a high risk of alveolar disorders. Matrix-degrading metalloproteinases are key regulators of lung remodeling.

In this study of premature neonates at a high risk of chronic lung disease, we measured gelatinase activities in airway secretions early in the course of hyaline membrane disease. These activities were then interpreted according to clinical outcome, focusing on subsequent development of BPD. We found that hyaline membrane disease was associated with an increase in MMP-9 level, the latter correlating with inflammatory cell recruitment to the airways; the increase in MMP-9 level was, however, not associated with subsequent onset of BPD. By contrast, low MMP-2 level was associated with later BPD onset, suggesting that MMP-2 level is essential for lung development and/or pulmonary tissue repair.

MMP-9 is a metalloproteinase secreted by a wide variety of cell types. In the lung, MMP-9 is synthesized by normal resident structural and inflammatory cells such as bronchial epithelial cells (37), alveolar epithelial cells (12, 28), and alveolar macrophages (35). All of these cell types can greatly increase their MMP-9 secretion after stimulation (12, 28, 35, 37). Furthermore, neutrophils recruited into alveoli during lung injury can also secrete large amounts of MMP-9 (17). MMP-9 has beneficial roles, such as cell spreading and migration during repair of the respiratory epithelium (21). However, most studies have focused on the potential deleterious effect of an imbalance between MMP-9 and TIMP induced by insults, and MMP-9 has been implicated in the pathogenesis of inflammatory airway diseases, such as cystic fibrosis (11), chronic bronchitis (33), and asthma (22, 33).

Our study provides evidence of MMP-9 level in the airways of premature neonates with hyaline membrane disease. Immediately after birth, MMP-9 levels were strongly influenced by the degree of prematurity, suggesting that maturation processes regulate the capacity of resident lung cells to secrete MMP-9. Our laboratory has previously found that spontaneous MMP-9 secretion by alveolar macrophages in newborn rats significantly varied with the lung growth phase (10). The number of alveolar macrophages is expected to be very low in premature neonates (10, 38), as confirmed here, and this may also contribute to the lower MMP-9 levels observed with increasing prematurity. Hyaline membrane disease was associated with increased MMP-9 level in the airways on days 3 and 5 of life; this level correlated with the number of inflammatory cells, suggesting that MMP-9 mainly originated from recruited, activated alveolar macrophages and neutrophils. In very premature neonates, the initially low MMP-9 activities increased on day 3 or 5, reaching levels at least as high as those observed in less premature infants, arguing for a similar ability to respond to lung injury. Once secreted, MMP-9 can be activated by many products present in inflammatory airways, especially oxidants (29) and neutrophil elastase (34). In our study, the presence and amount of...
activated MMP-9 were not related to BPD outcome. Thus MMP-9 may be considered as an important marker of the initial inflammatory response, but its contribution to the development of long-term sequel is uncertain. These findings are in complete agreement with our laboratory’s recent experiments performed in newborn rats (14). Indeed, we found that alveolar growth disorders induced by intratracheal instillation of lipopolysaccharide were associated with alveolar neutrophil influx and increased MMP-9 level, but we failed to prevent growth disorders by inhibiting MMP-9 activity. Thus, in newborn rats as in human preterm infants, no causal link could be established between MMP-9 overactivity induced by inflammatory processes and lung growth disorders.

MMP-2 detected in tracheal aspirate supernatants may originate from several cell types. Fibroblasts are usually considered as the main source of MMP-2 (27). Alveolar epithelial cells (12) and bronchial epithelial cells (37) can also secrete MMP-2. In fetal rats, both lung epithelial cells and fibroblasts express 72-kDa type IV collagenase, corresponding to MMP-2 (30). MMP-2 was shown to play a major role in the rapid degradation of type IV collagen, which occurs during the growth phase of late fetal lung development (2). MMP-2 may thus be an important regulatory factor for harmonious lung growth, and our results suggest that the activity detected in tracheal aspirate supernatants reflects this role. Total MMP-2 level measured on day 0 in tracheal aspirates was not significantly influenced by the degree of prematurity, suggesting that fetal lung cells are mature in terms of MMP-2 secretion. This result is in keeping with previous experimental data demonstrating that cultured human and rat fibroblasts of fetal, neonatal, or adult origin show comparable basal MMP-2 secretion (7, 18). Maturation processes may, however, influence MMP-2 activation mechanisms, as the fraction of total MMP-2 level accounted for by the activated form increased in neonates with higher gestational ages and birth weights. Experiments with mouse skin fibroblasts have also shown that MMP-2 spontaneously secreted by adult fibroblasts is partially activated, contrary to that secreted by fetal or neonatal cells (18).

In contrast to MMP-9, the MMP-2 level fell with time, and no correlation was found between MMP-2 levels and inflammatory cell counts, suggesting that lung insults and inflammatory airway responses do not stimulate MMP-2 secretion. However, this decrease with time must be interpreted with care, as it was observed in repeatedly sampled neonates who had the most severe hyaline membrane disease, i.e., requiring at least 3 or 5 days of mechanical ventilation. For obvious reasons, control samples could not be obtained from neonates weaned from the ventilator before day 3. Changes in MMP-2 level with time could, therefore, reflect either a decreased activity induced by injury, potentially interfering with lung development and/or repair, or a physiological transient increase activity induced by birth, as suggested by experiments in rats showing that lung type IV collagenase mRNA expression and type IV collagenolytic activity are highest before birth (23).

The lack of MMP-2 activity in bronchial lavage fluid in healthy adults and patients with status asthmaticus (22) underlines the specificity of MMP-2 profiles during the neonatal period and tend to confirm that MMP-2

**Fig. 3.** Changes in total MMP-2 level (A), total MMP-9 level (B), and tissue inhibitor of metalloproteinases (TIMP)-1 levels (C), measured from birth to day 3 or 5, in the 45 serially sampled neonates (change value = value at day 3 or 5 − value at day 0). Infants are divided into 2 subgroups according to subsequent development of BPD (defined as the persistence of oxygen supplementation at 36 wk postconception). Box plots for the median and 25th to 75th percentiles are shown; error bars span the 10th to 90th percentiles. Open plots, neonates without BPD; shaded plots, neonates with BPD. No significant difference was observed between the 2 subgroups.
levels in premature airways reflect physiological activity involved in postnatal matrix turnover.

The strong association that we found between low levels of MMP-2 on day 0 and BPD, independent of birth weight and FIO₂, further supports the link between MMP-2 level and postnatal lung growth. Low MMP-2 levels are thought to result in reduced lung proteolytic activity and impaired matrix remodeling. The fact that we did not observe simultaneous differences in soluble free gelatinolytic activity may be largely explained by the mechanisms required for MMP-2 activation.

Indeed, it was shown that MMP-2 activation was regulated at the cell surface and required a preliminary binding to a “receptor” complex formed by the membrane bound MT1-MMP and TIMP-2 (5). Furthermore, matrix proteolysis mediated by the activated MMP-2 was demonstrated to be very localized around the cells expressing MT1-MMP (13). Airway samples, which permit only the measurement of levels of soluble protein, may, therefore, poorly reflect the fraction of activated MMP-2 present at the cell surface into the lung tissue as well as the tissular proteolytic activity. Nevertheless, changes in pro-MMP-2 levels measured in airways may reflect coincident changes in matrix remodeling mediated by this protease, because in vitro experiments demonstrated that higher concentrations of pro-MMP-2 in cell culture medium were associated with greater areas of pericellular proteolysis in the underlying substrate (13). Although the reasons for this low MMP-2 level were not explored here, the very early occurrence of this profile after birth points to an intrinsic impairment. Such premature neonates may thus be unable to ensure normal lung development when faced with external insults.

TIMP-1 levels in our population were not associated with the initial severity of hyaline membrane disease or with the risk of BPD. TIMP-1 is a well-characterized specific inhibitor of MMPs. It interacts noncovalently with active metalloproteinases, including MMP-2 and MMP-9, and also binds to the proform of MMP-9, thus regulating its activation (26, 36). It is secreted by a wide variety of cell types, including lung epithelial cells, fibroblasts, alveolar macrophages, and neutrophils. Measurement of TIMP-2 levels might also be of value, as TIMP-2 also binds specifically to the proform of MMP-2 and participates in the regulation of MMP-2 activation. Unfortunately, most samples collected in our study were too small for TIMP-2 assay. During lung development, TIMP-1 mRNA expression in baboons (25) was low during fetal development but underwent a marked increase in the lung shortly after both premature and term birth. Our results are in keeping with this study, showing a postnatal increase in TIMP-1 levels in the airways, independent of gestational age. The coincident increases in TIMP-1 and MMP-9 levels may strongly contribute to the fact that activated MMP-9 was present at day 3 or 5 only in a minority of infants.

In conclusion, both MMP-2 and MMP-9 activities were found in airways of premature neonates with hyaline membrane disease. However, clear differences were found between these two metalloproteinases, regarding the influence of birth weight, changes induced by lung insult, and their role in the subsequent onset of BPD. MMP-9 level was influenced by birth weight and the inflammatory response to lung insult. No association was found with subsequent respiratory outcome. In contrast, the MMP-2 level was independent of birth weight and the local inflammatory response. Low MMP-2 level was strongly associated with subsequent onset of BPD.

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