ORIGINAL ARTICLE

Intrauterine neutrophil activation is associated with pulmonary haemorrhage in preterm infants

R Mehta, A Petrova

Arch Dis Child Fetal Neonatal Ed 2006;91:F415–F418. doi: 10.1136/adc.2005.092288

See end of article for authors' affiliations

Correspondence to: Professor Mehta, Department of Pediatrics, MEB 238, Robert Wood Johnson Medical School-UMDNJ One Robert Wood Johnson Place, New Brunswick, NJ 08903, USA; mehtara@umdnj.edu

Accepted 9 May 2006
Published Online First
16 May 2006

Background: Clinical and experimental studies showing lung damage associated with biologically active neutrophil derivatives suggests the possibility that intrauterine neutrophil activation may predispose to the development of pulmonary haemorrhage in extremely low birthweight infants early after birth.

Objectives: To assess neutrophil functional activity in cord blood from extremely low birthweight infants who subsequently developed severe pulmonary haemorrhage.

Methods: Neutrophil functional activity was evaluated in the cord blood of preterm neonates (gestational age <28 weeks and weight <800 g) who developed pulmonary haemorrhage ($n = 6$) and in controls who did not ($n = 6$). Infants with congenital abnormalities and noteworthy maternal complications such as diabetes, pre-eclampsia, haemorrhagic disorders, and connective tissue disease were excluded. Neutrophils isolated from cord blood immediately after delivery were tested to evaluate neutrophil chemotaxis, random motility, and chemiluminescence. Standard methodology was used.

Results: Chemotaxis and chemiluminescence of neutrophils from cord blood of infants with pulmonary haemorrhage was higher than in the controls ($34.8 (2.0) \vee 28.6 (0.8) \mu\text{m}$, $p < 0.001$, and $95.8 (8.5) \vee 73.2 (3.6) \text{ cpm} \times 10^{-3}$, $p < 0.001$ respectively). Random motility of cord blood neutrophils was comparable in the two groups ($28.6 (1.2) \vee 25.3 (1.2) \mu\text{m}$, $p < 0.09$).

Conclusions: Intrauterine induction of chemotactic activity and metabolic oxygenation of neutrophils may predispose to the development of pulmonary haemorrhage in extremely low birthweight infants with respiratory distress early after birth.

Pulmonary haemorrhage (PH) is an acute catastrophic event that occurs in 10–16% of extremely low birthweight (ELBW) preterm infants at a mean age of 38 hours.¹ Between 30% and 60% of infants with PH usually die within 72 hours of birth,^{1, 2} and 60% of those who survive develop chronic lung disease.²

The pathophysiology of PH in ELBW infants is still not clear.³ Their pulmonary structural tissue (bronchial, alveolar, and capillary) is often injured as the result of hypoxic or oxidative stress, inflammation, and mechanical insults.⁴ Hypoxia contributes to the development of pulmonary oedema and increased pulmonary microvascular pressure, factors that subsequently lead to PH in preterm infants. A lower capillary transmural pressure is needed to induce breaks in the capillary endothelial and alveolar epithelial membranes of newborn infants compared with adults.⁵ In addition, inflammation may exacerbate many types of lung injury.⁶ Neutrophils have been shown to play an essential role in initiating lung injury in preterm infants.^{7, 8} Neutrophil activation is associated with the release of biologically active products such as elastase and myeloperoxidase,^{8, 9} which are able to hydrolyse the components of the extracellular matrix and surfactant proteins^{10, 11} and induce acute injury of lung tissue.^{12, 13} An experimental study has shown that neutrophil elastase participates in the development of PH.¹⁴ Neutrophil activation associated production of reactive oxygen species has also been implicated as a causal factor in lung injury of antioxidant deficient preterm infants.¹⁵ To date, neutrophil functional activity has not been studied in ELBW infants who develop PH early after birth.

On the basis of evidence that neutrophil derivatives induce acute lung injury, we hypothesised that activation of neutrophils in the cord blood is associated with the development of PH in ELBW infants during the early neonatal period.

MATERIALS AND METHODS

Neutrophil functional activity was evaluated in cord blood obtained from six preterm neonates diagnosed with severe PH within the first 2–3 days (2.3 (0.5) days) of life (PH+). The diagnosis of PH was based on strong radiographic and clinical evidence. The radiographic findings ranged from patchy infiltrates to complete opacification of lung fields and pleural effusions. Clinical symptoms included oozing of bloody fluid from the nose and mouth or endotracheal tube associated with rapid worsening of respiratory status, cyanosis, and, in severe cases, shock.

The PH+ neonates were born at a gestational age of <28 weeks and weighing <800 g, and were therefore categorised as ELBW infants. Placental histology reports of all the infants diagnosed with PH showed acute chorioamnionitis. The cord blood neutrophils of six ELBW infants without pulmonary haemorrhage (PH–) and six healthy term infants were used as controls. The inclusion criteria for the preterm controls were histological chorioamnionitis, gestational age <28 weeks, and birth weight <800 g, but no clinical evidence of PH. The number of ELBW infants (with and without PH) included in this study allowed the identification of at least 30% difference between the mean of the tested variables of neutrophil functional activity, with a statistical power of 0.90 and $\alpha < 0.05$ (two sided). The general exclusion criteria for the cases and controls were congenital abnormalities, noteworthy maternal disease and complications during pregnancy and delivery such as diabetes, pre-eclampsia, haemorrhagic disorders, and connective tissue disorders, and drug use.

The Institutional Research and Publications Review Committee in Human Subjects approved the cord blood

Abbreviations: ELBW, extremely low birthweight; PDA, patent ductus arteriosus; PH, pulmonary haemorrhage

Table 1 Comparison of relevant data between extremely low birthweight infants who did and did not develop pulmonary haemorrhage

	PH+	PH–
Gestational age (weeks)	25 (0.9)	25 (0.8)
Birth weight (g)	680 (73)	697 (86)
Apgar (1 minute)	5.0 (1.4)	5.1 (2.7)
Apgar (5 minutes)	7.3 (0.5)	7.3 (1.0)
PDA diagnosed after birth	4 (67%)	3 (50%)
Indomethacin used after birth	4 (67%)	3 (50%)
Surfactant administered	6 (100%)	6 (100%)
Ventilation variables		
PIP	14.6 (1.0)	14.6 (1.0)
PEEP	4.8 (0.4)	4.8 (0.4)
FiO ₂	0.45 (0.1)	0.40 (0.1)
Rate	31.0 (5.3)	30.0 (6.3)
CBC during first 24 hours of life		
WBC ($\times 10^3/\text{mm}^3$)	8.4 (2.0)	11.0 (2.0)
PMN (%)	52.7 (8.6)	54.2 (6.2)
Bands (%)	2.0 (0.6)	1.7 (1.0)
Platelets ($\times 10^3/\text{mm}^3$)	145.3 (18.6)	139.3 (17.6)

Values are mean (SD) or number (%). There are no significant differences between the two groups.

PH+, Developed pulmonary haemorrhage; PH–, did not develop pulmonary haemorrhage; PDA, patent ductus arteriosus; PIP, peak inspiratory pressure; PEEP, peak end expiratory pressure; FiO₂, fractional inspired oxygen; CBC, complete blood count; WBC, white blood count; PMN, polymorphonuclear leucocytes.

studies. Informed consent was not required for the collection of cord blood. Neonatal cord blood was obtained immediately after delivery for neutrophil isolation and testing of neutrophil random motility, chemotaxis, and chemiluminescence.

Neutrophil isolation

Neutrophils were isolated from the heparinised blood samples by the method of Boyum¹⁶ and washed twice in Hanks' balanced salt solution without Ca²⁺, Mg²⁺, and phenol red (Sigma Laboratories, St Louis, Missouri, USA). After separation, the cell suspension contained 85% neutrophils, and trypan blue dye showed >95% neutrophil viability. The neutrophils were adjusted to a concentration of 2.0×10^6 cells/ml with 2% bovine serum albumin for chemotaxis and random motility, and 1.0×10^6 cells/ml in Hanks' balanced salt solution for the chemiluminescence studies.

Measurement of chemotaxis and random motility

Chemotaxis and random motility were quantified using modified Boyden microchemotactic chambers (Neuro-Probe Inc, Bethesda, Maryland, USA).¹⁷ The migration through 5 μm pore cellulose nitrate filters (Millipore Corp, Bedford, Massachusetts, USA) was measured in response to pooled human adult serum activated by zymosan (Sigma) for chemotaxis, or tissue culture medium 199 (Gibco Laboratories, Grand Island, New York, USA) for random motility, in the lower wells. The chambers were incubated (two hours, 37°C) and then analysed by an investigator blinded to the sample source. Chemotaxis and random motility are expressed as the mean depth (μm) at which the leading front of neutrophils was in focus using the $\times 40$ objective lens. Values represent the mean of five randomly chosen microscopic fields in each filter. Using this assay, the coefficient of variation between assays for healthy adult neutrophils was <10%.

Measurement of chemiluminescence

Luminol enhanced chemiluminescence (Sigma Diagnostics, St Louis, Missouri, USA), a measure of energy released as photons during oxidative metabolism,¹⁸ was measured using a liquid scintillation system (Beckman model LS-230; Beckman Instruments Inc, Fullerton, California, USA).

Latex particles (0.8 μm in diameter; Seragon Diagnostics, Indianapolis, Indiana, USA) functioned as stimulants for the phagocytosis and oxidative burst. Peak levels in counts per minute (cpm $\times 10^3$) for 1.0×10^6 neutrophils were quantified in duplicate.

Statistical analysis

Measurements of neutrophil functional activity are presented as mean (SD). Statistical differences were evaluated using analysis of variance followed by the Tukey test. Categorical data were compared by the χ^2 test. A two tailed p value of <0.05 was considered significant.

RESULTS

No significant differences in sex, gestational age, and birth weight (which was appropriate for gestational age for all the infants) were found between the cases (PH+) and controls (PH–) (table 1). The cases and controls were comparable with respect to (a) variables of ventilatory assistance provided after birth, (b) indomethacin and surfactant treatment, (c) the proportion of infants diagnosed with large patent ductus arteriosus (PDA), and (d) complete blood count results obtained during the first 24 hours.

None of the infants in this study (cases or controls) was diagnosed with early onset culture confirmed sepsis. There were three neonatal deaths: two in the PH+ group from the PH, and one in the PH– group from complications of necrotising enterocolitis and late onset sepsis.

Figure 1 presents a comparison of neutrophil chemotaxis, random motility, and chemiluminescence between the cases and controls. Overall, they were significantly reduced in both groups of ELBW infants (PH+ and PH–) compared with term controls. Chemotaxis and chemiluminescence of neutrophils isolated from the cord blood of PH+ infants were higher than in PH– controls (34.8 (2.0) v 28.6 (0.8) μm , $p < 0.001$, and 95.8 (8.5) v 73.2 (3.6) cpm $\times 10^3$, $p < 0.001$ respectively). The difference in random motility did not reach significance (28.6 (1.2) v 25.3 (1.2) μm , $p < 0.09$).

DISCUSSION

For the first time, we have evaluated cord blood neutrophil functional activity of ELBW infants who subsequently developed severe PH at an early postnatal age. We have shown significant association between PH morbidity and activated cord blood neutrophils in infants with PH, whereas the known factors that predispose to PH, such as gestational age, birth weight, severity of PDA, and exogenous surfactant therapy,¹⁹ were similar to those in controls (PH–).

Increased cord blood neutrophil chemotaxis and chemiluminescence in the infants with PH reflects aggravation of neutrophil migration and induction of neutrophil metabolic oxygenation, which accelerates oxidative stress and apoptosis and leads to acute lung injury.^{20–21} A human and animal model revealed an important causative role for neutrophils in acute lung injury²² and the development of PH.¹⁴ Neutrophils contribute to the pathogenesis of respiratory distress after premature birth.²³ The pathogenesis of PH in newborn infants with respiratory distress syndrome is associated with the entrapment of neutrophils in the pulmonary microcirculation which initiates and amplifies the injury to the alveolar capillary membrane, disturbs capillary blood flow, enhances pulmonary permeability, and causes advanced pulmonary oedema.²⁴ Neutrophil proteases and reactive oxygen species products have been shown to play a pivotal role in pulmonary and vascular tissue injury.^{25–26} In clinical practice, exacerbation of lung problems such as longer duration of ventilatory support and oxygen requirement in preterm infants is associated with absolute neutrophil counts $> 30 \times 10^3/\text{mm}^3$.²⁷ A study that showed the beneficial effect of direct inhibition of neutrophil

Figure 1 Comparison of neutrophil chemotaxis (A), random motility (B), and chemiluminescence (C) between extremely low birthweight infants who developed pulmonary haemorrhage (PH+) and those who did not (PH-).

activation in the prevention of acute lung pathology²⁸ may confirm the pathogenic role of neutrophil activation in lung pathology.

The next question that arises is what factors contribute to the activation of cord blood neutrophils in ELBW infants. In general, a variety of molecules are known to stimulate neutrophil activation,²⁹ and some, such as interleukin 8 and monocyte chemoattractant protein 1, have been found to be increased in preterm infants with PH.³⁰ Experimental studies have shown that increased concentrations of vascular endothelial growth factor stimulate chemotaxis³¹ and induce PH in preterm mice.³² Interestingly, the role of infection modelling by lipopolysaccharide in the development of PH has not been confirmed.²⁸ Because of reduced expression of lipopolysaccharide binding proteins by neutrophils from newborns, lipopolysaccharide does not upregulate them effectively.³³ A recent publication reported that anti-neutrophil cytoplasmic autoantibodies contribute to PH in neonates

born to mothers with systemic vasculitis³⁴ by stimulating neutrophil functions such as generation of reactive oxygen species, degradation of protease release,³⁵ and chemotactic activity.³⁶ In this study, we did not evaluate the factors that may have activated the neutrophils in the cord blood of the ELBW infants presenting with PH in the early neonatal period, and this could be a subject for further research. However, we conclude that the significant increase in cord blood neutrophil chemotaxis and chemiluminescence promotes development of PH in ELBW infants with respiratory distress syndrome early after birth.

Authors' affiliations

R Mehta, A Petrova, Department of Pediatrics, Division of Neonatal Medicine, Robert Wood Johnson Medical School-UMDNJ, New Brunswick, New Jersey, USA

Competing interests: None declared.

What is already known on this topic

- Factors that predispose to pulmonary haemorrhage in preterm neonates include gestational age, birth weight, severity of PDA, and exogenous surfactant therapy

What this study adds

- Intrauterine neutrophil activation may predispose to the development of pulmonary haemorrhage in extremely low birthweight infants with respiratory distress early after birth

REFERENCES

- 1 Kluckow M, Evans N. Ductal shunting, high pulmonary blood flow, and pulmonary hemorrhage. *J Pediatr* 2000;**137**:68–72.
- 2 Pandit PB, O'Brien K, Asztalos E, et al. Outcome following pulmonary haemorrhage in very low birthweight neonates treated with surfactant. *Arch Dis Child Fetal Neonatal Ed* 1999;**81**:F40–4.
- 3 Braun KR, Davidson KM, Henry M, et al. Severe pulmonary hemorrhage in the premature newborn infant: analysis of presurfactant and surfactant eras. *Biol Neonate* 1999;**75**:18–30.
- 4 Zoban P, Cerny M. Immature lung and acute injury. *Physiol Res* 2003;**52**:507–16.
- 5 Fu Z, Heldt GP, West JB. Increased fragility of pulmonary capillaries in newborn rabbit. *Am J Physiol Lung Cell Mol Physiol* 2003;**284**:L703–9.
- 6 Strieter RM, Kunkel SL, Keane MP, et al. Chemokines in lung injury: Thomas A. Neff Lecture. *Chest* 1999;**116**:103S–10S.
- 7 Jobe AH, Ikegami M. Mechanisms initiating lung injury in the preterm. *Early Hum Dev* 1998;**53**:81–94.
- 8 Merritt TA, Cochrane CG, Holcomb K, et al. Elastase and alpha 1-proteinase inhibitor activity in tracheal aspirates during respiratory distress syndrome: role of inflammation in the pathogenesis of bronchopulmonary dysplasia. *J Clin Invest* 1983;**72**:656–66.
- 9 Nakamura H, Yoshimura K, McElvaney NG, et al. Neutrophil elastase in respiratory epithelial lining fluid of individuals with cystic fibrosis induces interleukin-8 gene expression in a human bronchial epithelial cell line. *J Clin Invest* 1992;**89**:1478–84.
- 10 McDonald JA, Kelly DG. Degradation of fibronectin by human leukocyte elastase: release of biologically active fragments. *J Biol Chem* 1980;**255**:8848–58.
- 11 Liao DF, Yin NX, Huang J, et al. Effects of human polymorphonuclear leukocyte elastase upon surfactant proteins in vitro. *Biochim Biophys Acta* 1996;**1302**:117–28.
- 12 Janoff A. Elastase in tissue injury. *Annu Rev Med* 1985;**36**:207–16.
- 13 Weiss SJ. Tissue destruction by neutrophils. *N Engl J Med* 1989;**320**:365–76.
- 14 Cantin AM. DNase I acutely increases cystic fibrosis sputum elastase activity and its potential to induce lung hemorrhage in mice. *Am J Respir Crit Care Med* 1998;**157**:464–9.
- 15 Saugstad OD. Oxygen toxicity in the neonatal period. *Acta Paediatr Scand* 1990;**79**:881–92.
- 16 Boyum A. Isolation of leucocytes from human blood. Further observations. Methylcellulose, dextran, and ficoll as erythrocyteaggregating agents. *Scand J Clin Lab Invest Suppl* 1968;**97**:31–50.
- 17 Smith CW, Hollers JC, Patrick RA, et al. Motility and adhesiveness in human neutrophils. Effects of chemotactic factors. *J Clin Invest* 1979;**63**:221–9.
- 18 Stevens P, Winston DJ, Van Dyke K. In vitro evaluation of opsonic and cellular granulocyte function by luminol-dependent chemiluminescence: utility in patients with severe neutropenia and cellular deficiency states. *Infect Immunol* 1978;**22**:41–51.
- 19 Raju TN, Langenberg P. Pulmonary hemorrhage and exogenous surfactant therapy: a meta analysis. *J Pediatr* 1996;**123**:603–10.
- 20 Lundqvist-Gustafsson H, Bengtsson T. Activation of the granule pool of the NADPH oxidase accelerates apoptosis in human neutrophils. *J Leukoc Biol* 1999;**65**:196–204.
- 21 Abraham E. Neutrophils and acute lung injury. *Crit Care Med* 2003;**31**:S195–9.
- 22 Lee WL, Downey GP. Neutrophil activation and acute lung injury. *Curr Opin Crit Care* 2001;**7**:1–7.
- 23 Carlton DP, Albertine KH, Cho SC, et al. Role of neutrophils in lung vascular injury and edema after premature birth in lambs. *J App Physiol* 1997;**83**:1307–17.
- 24 Cole VA, Normand IC, Reynolds EO, et al. Pathogenesis of hemorrhagic edema and massive pulmonary hemorrhage in the newborn. *Pediatrics* 1973;**51**:175–87.
- 25 Westlin WF, Gimbrone MA Jr. Neutrophil-mediated damage to human vascular endothelium. Role of cytokine activation. *Am J Pathol* 1993;**142**:117–28.
- 26 Pober JS, Cotran RS. The role of endothelial cells in inflammation. *Transplantation* 1990;**50**:537–44.
- 27 Hsiao R, Omar SA. Outcome of extremely low birth weight infants with leukemoid reaction. *Pediatrics* 2005;**116**:e43–51.
- 28 Murakami K, Okajima K, Uchida M. The prevention of lipopolysaccharide-induced pulmonary vascular injury by pretreatment with cephalexin in rats. *Am J Respir Crit Care Med* 2000;**161**:57–63.
- 29 Condliffe AM, Kitchen E, Chilvers ER. Neutrophil priming: pathophysiological consequences and underlying mechanisms. *Clin Sci (Lond)* 1998;**94**:461–71.
- 30 Baier RJ, Loggins J, Kruger TE. Increased interleukin-8 and monocyte chemoattractant protein-1 concentrations in mechanically ventilated preterm infants with pulmonary hemorrhage. *Pediatr Pulmonol* 2002;**34**:131–7.
- 31 Clauss M, Weich H, Breier G, et al. The vascular endothelial growth factor receptor Flt-1 mediates biological activities. Implications for a functional role of placenta growth factor in monocyte activation and chemotaxis. *J Biol Chem* 1996;**271**:17629–34.
- 32 Le Cras TD, Spitzmuller RE, Albertine KH, et al. VEGF causes pulmonary hemorrhage, hemosiderosis, and air space enlargement in neonatal mice. *Am J Physiol Lung Cell Mol Physiol* 2004;**287**:L134–42.
- 33 Qing G, Howlett S, Bortolussi R. Lipopolysaccharide binding proteins on polymorphonuclear leukocytes: comparison of adult and neonatal cells. *Infect Immun* 1996;**64**:4638–42.
- 34 Bansal PJ, Tobin MC. Neonatal microscopic polyangiitis secondary to transfer of maternal myeloperoxidase-antineutrophil cytoplasmic antibody resulting in neonatal pulmonary hemorrhage and renal involvement. *Ann Allergy Asthma Immunol* 2004;**93**:398–401.
- 35 Falk RJ, Terrell RS, Charles RA, et al. Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals in vitro. *Proc Natl Acad Sci USA* 1990;**87**:4115–19.
- 36 Keogan MT, Esnault VL, Green AJ, et al. Activation of normal neutrophils by anti-neutrophil cytoplasm antibodies. *Clin Exp Immunol* 1992;**90**:228–34.