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Cell death and lung cell histology in meconium aspirated newborn rabbit lung

Received: 23 November 1999 and in revised form: 12 March and 9 May 2000 / Accepted: 10 May 2000

Abstract Meconium aspiration syndrome (MAS) is a major cause of newborn mortality and morbidity. In this study we investigated the inflammatory responses and morphological changes in the newborn lung to debris-free meconium instillation. We developed a model for studies of MAS using 2-week-old rabbit pups. Cell death was assessed by DNA staining and detection of DNA fragmentation by in situ end labeling. Cell death was seen in association with an increase of inflammatory cytokines levels, studied by ELISA. Necrotic cells were detected by staining of lavage cells with ethidium bromide and 4',6'-diamino-2'-phenyldion. Meconium instillation resulted selectively in loss of airway and alveolar epithelial cells followed by cell death, which increased with time. Necrotic cells looked smaller and damaged with maximal counts at 24 h after instillation.

Conclusion Meconium instillation into lungs caused massive cell death, possibly by apoptosis, and necrosis that may have been activated by the inflammatory cytokine production.

Key words Cell death · Cytokines · In situ DNA end labeling · Lungs · Meconium

Abbreviations DAPI 4',6'-diamino-2'-phenyldion · IL interleukin · ISEL in situ DNA end labeling · MAS meconium aspiration syndrome · PBS phosphate buffer solution · PGE₂ prostaglandin E₂ · TNFα tumor necrosis factor α

Introduction

The pathogenesis of meconium aspiration syndrome (MAS) is under active investigation. Several animal models have been used for the study of MAS, namely lambs [13], piglets [18], rats [1] and calves [10]. These studies have identified multiple mechanisms of lung injury, namely airways obstruction, surfactant inactivation [1, 18], pulmonary inflammation, decreased arterial oxygen tension and increased pulmonary hypertension. There is a large body of evidence regarding the role of hypoxia and pulmonary hypertension in the development of clinical MAS. The role of meconium induced inflammation is less well known.

Tyler et al. [15] found mechanical damage of the lung tissue, alveolar and airway obstruction and chemical pneumonitis 48 h after instillation of 20% fresh human meconium into adult rabbit lungs. Histological analysis of lung specimens in the lamb model of MAS showed homogeneously expanded alveoli with intact alveolar epithelium, acute interstitial inflammation and vasoconstriction induced by hypoxia.

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Intense inflammatory reaction in the lungs was accompanied by extensive accumulation of neutrophils, T-lymphocytes, monocytes and macrophages [12, 20]. The concentrations of potent neutrophil chemotactants increased in bronchoalveolar lavage fluid from patients with pulmonary disease. Elevated levels of tumor necrosis factor α (TNF-α), interleukin (IL) 6 and 8 also have been found in infants after meconium aspiration and hyaline membrane disease [7, 15].

In this study we hypothesized that soluble fraction of human meconium initiated pulmonary inflammatory response that leads to the development of MAS.

Materials and methods

Meconium preparation

Meconium was prepared according to a previously published procedure [18]. First pass human meconium samples were obtained from full-term, healthy human neonates. It was then pooled, homogenized, filtered before the experiment and diluted with sterile saline. Fresh meconium (1 g) was homogenized on ice in a blender with 9 ml of 0.9% NaCl to a 10% (weight/volume) final concentration and was spun down at 5,000 rpm for 20 min (4 °C) to separate supernatant and pellet. The supernatant was filtered via a glass filter followed by sterilization via a 0.2 μm filter (both filters were from Millipore Co., Bedford, Mass.). This debris-free supernatant was used in the study.

Animal model

New Zealand white rabbit pups, 2 weeks old, (LSP Industries, Union Grove, Wis., USA) were used in the study. The animals were handled according to the National Institute of Health guidelines. They were housed with the mother for few days before the experiment in stainless steel rabbit cages. The mothers were given regular Purina rabbit chow (Scientific Animal Feed Co., Arlington Heights, Ill., USA). The Animal Care and Use Committee of Michael Reese Hospital, Chicago, approved the experimental protocol. Prior to meconium instillation, rabbits were lightly anesthetized with an intraperitoneal injection of 10 mg/kg ketamine and 1 mg/kg xylazine. A small midline incision was made on the ventral aspect of the neck to expose the trachea and an endotracheal tube was placed through the incision, then stabilized. After 15 min, 10% sterile meconium supernatant (1.2 ml/kg) was instilled through the endotracheal tube followed by a 5 ml bolus of air to disperse the meconium into the lungs. Skin and tracheal incisions were then closed with 4-0-nylon suture and pups were allowed to breathe spontaneously in room air. Different groups of pups were sacrificed using nembutal (100 mg/kg, i.p.) at 0, 2, 4, 8 and 24 h after meconium injection. A total of 50 rabbit pups were used for the study. 25 meconium injected and 25 controls. Control rabbit pups received equivalent volumes of 0.9% NaCl, instead of meconium and were sacrificed at the same five different time points mentioned above. Immediately following sacrifice, the chest was opened by a midline incision, lungs were isolated and lung lavage was performed. The lavage fluid was used to study alveolar cell death and cytokine levels.

Studies of meconium distribution in lungs

Prior to initiating the above mentioned studies we studied the dispersion of meconium in the lungs by instillation of a meconium/fluorescent mixture (one part fluorescent solution – FITC labeled bovine serum albumin, Sigma Chemical Co., St. Louis, Mo., USA, 0.1 μg/ml and nine parts 10% meconium supernatant) in two rabbits. Then lungs were isolated and flash frozen in liquid nitrogen.

The distribution of meconium in lungs was assayed under ultraviolet light (yellow) and compared to un.injected lungs (pink). These observations confirmed uniform spread of meconium in the lungs.

Bronchoalveolar lavage and recovery of lung cells

Bronchial lavage was performed according to a previously published method [8]. After the sacrifice of each rabbit, the trachea was cannulated and lungs were isolated. The lung was lavaged through the main stream bronchus tube using 5 ml aliquots of 37 °C preheated phosphate buffered solution complemented with 20 mM HEPES (pH 7.3) in order to wash out alveolar cells. This buffer helps to keep lavage cells alive for a few hours. The procedure was repeated six times to recover a total of 30 ml of lavage fluid. These aliquots were pooled and processed immediately.

DNA staining and cell morphology

DNA staining can differentiate macrophages and neutrophils. Nuclei of macrophages are round in form but nuclei of neutrophils do not have any form. The DNA staining allowed us to stain entire nuclei and differentiate macrophages and neutrophils. Lung aspirates were spun down at 1,500 rpm for 5 min at room temperature and cell pellets were collected. Lavage cells were washed three times with PBS and counted. Then approximately 10,000 lavage cells were plated and dried on the surface of a microscopic glass plate. Cells were fixed in methanol/acetic acid (3:1) solution for 5 min, dried at room temperature for 10 min, washed twice in PBS (1 min per wash), and stored at 4 °C. Prior to fluorescent analysis, 10 μl of 0.1 μg/ml DNA-specific fluorescent dye (DAPI, Sigma Chemical Co.) was applied to plate. After DNA staining, damaged nuclei were identified using fluorescent microscopy (Olympus BX-60 fluorescent microscope, Japan).

Cell death

Lung aspirates were collected as described above, centrifuged, and washed twice in prewarmed (37 °C) PBS. Then lavage cells were suspended in the same buffer to a concentration of 10,000 cells/ml. An equal volume of ethidium bromide/acridine orange solution (50 mg ethidium bromide and 15 mg acridine orange in 1 ml 95% ethanol) was added to differentiate cell death. A 1% solution of this mixture in H2O was homogenized with same volume of lung lavage cells and then examined under a fluorescent microscope. Live and dead cells were detected by fluorescence, live cells stained with acridine orange appearing green and dead cells stained with ethidium bromide appearing red [11, 19].

Lung histology

The right mainstream bronchus of the lung was cannulated and instilled with 4 ml of 4% buffered formaldehyde solution which was introduced via a feeding tube for 1 h from a reservoir at a height of 20 cm. The bronchus was ligated and the lung tissue was fixed in the same solution for an additional 15 h at 4 °C, then the lung was sectioned using a table microtome (Zeiss Co., Lukas Microscope Service Inc., Skokie, Ill., USA). Basic staining with hematoxylin-eosin (H&E) of freshly resected lungs was performed and analyzed under magnification of a 50 and 100 μm/cm by light microscopy.

DNA fragmentation assay

Tissue processing

The right lobe specimens of rabbit lung tissue were fixed in buffered 4% formaldehyde for at least 15 h and embedded in paraffin.