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Age-dependent changes in porcine alveolar macrophage function during the postnatal period of alveolarization

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Abstract

During early postnatal ontogeny in most mammals, the lung is structurally and functionally immature. In some species with relatively altricial lung morphology, there is evidence of a coupling between functional maturity of the pulmonary cellular immune system and alveolar maturation. Herein, we examine changes in alveolar macrophage (AM) number and function occurring during alveolarization in a more precocial species, the pig, to determine if heightened oxidative metabolism and phagocytic ability is similarly delayed until completion of lung morphogenesis. We assessed cell differential in lavage fluid and evaluated two main functional parameters of AM phagocytic response, the generation of reactive oxygen species (ROS), and particle internalization. AM functional maturation occurred mainly during the first postnatal week: the proportion of AMs, ROS generation, and phagocytosis all increased significantly. These results suggest maturational improvement of the impaired AM-based pulmonary immune system of the neonate piglet occurs during the postnatal period of rapid alveolarization.

Keywords

Lung; ontogeny; phagocytosis; ROS; bronchoalveolar lavage; neonate; pig; superoxide anion

1. Introduction

Early in the postnatal lung ontogeny of many mammals, the adult number of alveoli has not yet been attained and the pulmonary cellular defense system has not yet achieved peak ability. The ongoing development of the lung postnatally has implications for pulmonary susceptibility, as age is a critical determinant of the ability of the lung to deal with environmental stressors [1]. A number of mammals display evidence of compromised pulmonary defense systems during early postnatal development [2-13]. The heightened susceptibility to infection in the neonate could be a consequence of impairment in the quantity,

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function, and/or regulation of one or several of the components of the cellular pulmonary immune system [14].

Alveolar macrophages (AMs) are the primary phagocytes of the innate pulmonary immune system, and constitute a link between innate and adaptive immunity. The immune recognition function of macrophage surface receptors has been recently summarized [15]. Although the production of reactive oxidants by AMs is beneficial in killing invading microorganisms, it is potentially detrimental to nearby tissue [16,17]. Functional immaturity of AMs during postnatal development has been demonstrated in rats [7,12,18], a species in which the lung parenchyma is structurally immature at birth compared to many other animals. A coupling between functional maturity of the cellular defense system and morphological maturity of the lung parenchyma has been suggested: rat AMs attained full oxidant releasing capacity only once the major period of postnatal alveolar morphogenesis had been completed and, it is hypothesized, the lung tissue thus more resistant to oxidant stress [18]. Similarly, in human children, alveolarization proceeds through the first few years [19], and the AMs of children under two are less effective in reducing nitroblue tetrazolium (a measure of oxidant burst) than those of older children [20]. Conversely, in a more precocial species, the guinea pig, it has been argued that enhanced oxidant generation in the AMs of young juveniles may help compensate for their lower serum opsonic activity [21].

In the present study, we assess postnatal changes in phagocytic function in the AMs of pigs (*Sus scrofa*), a precocial species in which alveolar structure is relatively mature at birth [22]. The morphology and morphometry of the pig lung through postnatal ontogeny have been well investigated [22-24] and the majority of alveolarization is known to be completed within two weeks of age. Relatively little information exists, however, regarding the accompanying maturation of the pulmonary cellular immune system during this time. Here, we investigate AM functional maturation that accompanies the period of alveolarization by characterizing the bronchoalveolar lavage fluid (BALF) cellular content, phagocytic response, and release of reactive oxygen species (ROS) during the first two postnatal weeks. The results suggest that deficiencies in the ability of AMs to phagocytize and kill foreign microbes during the early postnatal period could contribute to the increased susceptibility to infection observed in neonates.

2. Materials and Methods

Reagents were obtained from Sigma Aldrich (St. Louis, MO) unless otherwise indicated.

2.1. Animals

Male Landrace swine were obtained from Ramundo's Ranch (Cañuelas, Pcia de Bs.As., Arg.). Animals were from herds with no history of respiratory disease. The pigs were conventionally housed until lung harvest at the following four ages: 2 days, 4 days, 1 week, and 2 weeks. The data from a minimum of 3-5 piglets per age group were used for each assay. Each animal was weighed and then anesthetized with sodium thiopental [315mg/ml, final volume: 3ml (Zoovet, San Luis, Arg.)]. Institutional and Argentinean national guidelines for the use and care of laboratory animals were strictly observed.

2.2. Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed as previously described [25,26]. The diaphragm was punctured from the abdominal side to collapse the lungs. The trachea was isolated and cannulated using tracheal PVC tubing (Rusch, GmbH, Germany) attached to a 20 ml syringe. The tubing diameter varied depending on the age of the animal. The excised lung was then gently massaged and lavaged using 20 ml of phosphate buffered saline (PBS, no calcium, no

magnesium, pH 7.2-7.4). PBS was infused and withdrawn over a period of 30 seconds. Seven washes were performed, and the amount of fluid recovered from each wash was recorded. The final volume of BALF obtained in every experiment was 150 ml, a volume containing a sufficient number of cells to run all required biochemical assays. Centrifuge tubes containing the BALF were stored on ice until spinning down at 800g for 10 min. at 4°C. Supernatant was removed and the cell pellet was re-suspended in serum-free Roswell Park Memorial Institute media (RPMI –1640).

2.3. Alveolar macrophage culture and viability

AMs harvested by bronchoalveolar lavage as described above were re-suspended in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin. Cell viability was assayed by the Trypan Blue dye exclusion test (>95%). In all experiments, AMs were plated in 35 mm plastic dishes at a density of $4.5\text{--}5 \times 10^5$ and were incubated for 20 min. at 37°C in 5% CO₂. After incubation, plates were carefully washed to remove any non-adherent cells. The remaining attached cells were >95% macrophages (as tested by 0.1% neutral red dye, 10 min.).

2.4. Measurement of superoxide anion by NBT reduction test

Age-dependent differences in the intracellular release of superoxide anion (O₂⁻) produced by phagocytes during the respiratory burst were evaluated through quantitation of blue formazan precipitate in the cells following NBT reduction, as previously described [27] and adapted by Tasat et al. [9]. Isolated pulmonary cells obtained from BALF were incubated with 1% NBT in PBS, in the presence or absence of the phorbol ester, TPA (12-O-tetradecanoylphorbol 13-acetate). TPA is a strong macrophage activator and known inductor of ROS generation. TPA was added to the cell suspension at a concentration of 0.5 µg/ml for 45 min. at 37°C under continuous agitation. The number of reactive and non-reactive cells was scored using light microscopy. The staining intensity was analyzed semi-quantitatively by light microscopy. The intensity of stain was characterized as negative (0; no color), light (+; pale blue), strong (++; blue) or very intense (+++; deep blue-violet). Three to four animals per age class, ~100 cells per individual, were evaluated and the percentage cells exhibiting each class of staining intensity was calculated.

2.5. Phagocytosis assay

Fluorescein isothiocyanate (FITC)-labeled polystyrene beads (2.2 µm in diameter; Molecular Probes, Eugene, OR) were opsonized by re-suspension and incubation in FCS for 1 h at 37°C. Cultured BAL cells were incubated in RPMI medium with a 1:10 cell-to-bead ratio for 2 h at 37°C. After incubation, AMs were washed twice in PBS to remove non-adherent beads and fixed in 0.5% paraformaldehyde. To distinguish strongly absorbed beads from ingested ones, parallel experiments were performed at 4°C, a temperature known to block phagocytosis. The phagocytosis percentage plotted represents the difference between the number of bead-associated cells at 37°C and 4°C. The fluorescent beads were visualized by epifluorescence (Axioplan Microscope, Carl Zeiss, Jena, Germany) and the numbers of phagocytic and non-phagocytic cells were counted. To assess phagocytic efficiency, the number of beads per AM was also determined, and binned as <5 beads/per cells, 5-10 beads/cell and >10 beads/cell. A minimum of 100 macrophages per plate, three plates per animal, three animals per age group, were analyzed.

2.6. Lung histology

To verify that lung parenchymal maturation of the animals used in our BALF and macrophage studies was consistent with that previously reported in the literature, tissue was harvested for histological examination. Prior to lavage, the lungs were carefully removed from the chest

cavity, and the wet weight recorded. Lungs were fixed by intratracheal instillation of 10% cold neutral buffered formalin, with the fixative maintained at a constant pressure of 25cm water above the mid-thorax to ensure preservation in an inflated state. At this pressure, the volume of fixative in the lung is expected to approach total lung capacity. The volume of fixative required was recorded and the lungs allowed to fix overnight prior to subsequent processing for paraffin embedding. Tissue samples for histology were taken from lung lobes that had not been lavaged. Mallory's connective tissue staining was used to label elastin and examine basic histomorphology. Representative digital images of lung parenchyma sampled from the medial portion of the upper right lobe at each age were captured using a Sony charge-coupled device (CCD) camera coupled to a Nikon microscope (Nikon Instruments, Melville, NY) and a Silicon Graphics workstation using IrisCapture software (SGI, Sunnyvale, CA). Images were acquired under a 20x objective lens using transmitted light from a tungsten light source. In some cases, the brightness of the entire field of view was adjusted, but no further image manipulation or processing was performed. As the morphometrics of the developing piglet lung have been comprehensively investigated elsewhere [22,28], we did not repeat quantitative characterization of postnatal lung morphology in this investigation.

CD68 Immunocytochemistry—Lung macrophages undergo immunophenotypic maturation typified by a changing distribution of certain macrophage differentiation antigens. We evaluated the expression of CD68, a maturational marker, in the postnatally developing pig lung. Lung tissue obtained from pigs of 2, 4, 7, and 14 days of age was immunohistochemically labeled with CD68 EBM11 (Dako, Carpinteria, CA). The EBM11 clone is known to react strongly and consistently with terminally differentiated tissue macrophages [29]. Methods for CD68 EBM11 labeling were adapted from Ackermann et al. [30] and Greywoode et al. [29]. Following deparaffinization and rehydration, antigen retrieval was performed using 0.1% trypsin at 37°C. After blocking, the tissue was labeled with monoclonal mouse anti-human CD68 (diluted 1:30 in buffer) and visualized with diaminobenzidine (DAB) using a biotin-streptavidin-based detection kit (Biogenex, San Ramon, CA). To evaluate nonspecific staining, controls were run using nonspecific immunoglobins in place of the primary antibody. Tissue sections were counterstained with Mayer's hematoxylin. Images were recorded under a 60x objective lens using a Nikon light microscope.

2.7. Statistical analysis

Data are reported as means with variability represented as error bars of ± 1 standard deviation. All means represent the data from 3-5 piglets from each age class. Differences among the four age groups were evaluated by analysis of variance (ANOVA) using Statistics Calculator 3.0 (Statpac Inc., Bloomington, MN) or Intercooled Stata (vers. 7, StataCorp. LP, College Station, TX) software. In posthoc comparisons among the groups, a p-value of <0.05 was considered to indicate a significant difference.

3. Results

3.1. Postnatal Growth of Lung

To verify that the piglets were growing normally during the course of the experiment, mean body weight and lung weight for each age group were recorded. As expected, the piglets exhibited rapid postnatal growth: mean body weight and lung weight both increased during the first two weeks, though not always proportionately (data not shown). During the age range examined, body weight increased from 2.1 ± 0.19 kg at 2-days to 5.4 ± 1.0 kg at 14 days, and lung weight from 47.3 ± 2.7 g to 95.5 ± 9.3 g.

Postnatal changes in parenchymal morphology were observed in tissue harvested from the same animals as were used in the biochemical assays. As anticipated [22], pig lung parenchyma displayed a high degree of structural maturity even in very young animals. The neonate piglet lung was already advanced past the primary saccular stage that is observed in newborn rodents; by 2 days, the lung was partially alveolated (Fig. 1) and displayed putative secondary septal buds with elastin fibers at the apical crests. Elongation of the septal buds and alveolarization occurred rapidly within the first week, and the airspaces became smaller with increasing subdivision. Immunohistochemistry for the macrophage maturational marker CD68 EBM11 produced positive granular cytoplasmic labeling in AMs in lung parenchymal tissue of all age classes (Fig. 1, insets). We did not detect any trends in the intensity or localization of staining with age. No specific staining was observed in the negative controls.

3.2. Age-dependent increase in the percentage of AMs in the BALF

Inter-individual variation in total cell count values from BALF was large and we found no statistically significant change in the total cell count/ml over the age ranges investigated. Similarly, when total cell count was normalized to lung wet weight, there was no significant difference across age groups (data not shown). Differential cell counts indicated that in all age groups, AMs were the predominant cell type present in the BALF (Fig. 2). The remaining cell types were predominantly polymorphonuclear neutrophils (PMNs). Lymphocytes represented <1% of total cells in the four age groups analyzed. The percent content of AMs in the BALF increased ~1.4-fold over the first postnatal week. Over the first two weeks, the wet weight of the lung more than doubles, thus although the percentage of AMs went up, the total number of AMs normalized per gram of lung tissue did not increase significantly. The percentage of PMNs decreased significantly over the first week, from 33% at 2 days to 6% at 7 days. PMNs normalized per gram of lung also decreased. During the second week, there was no appreciable change in the percentages of cells that were AMs or PMNs.

3.3. Age-dependent ability of AMs in the resting state to produce superoxide anion

The antimicrobial activity of macrophages is due both to oxidative and non-oxidative processes. When AMs are stimulated, their oxidative metabolism increases, with superoxide anion and hydrogen peroxide being the main oxygen species being produced. Superoxide anion generation during the respiratory burst was evaluated using the Nitro Blue Tetrazolium (NBT) reduction test. In unstimulated AMs (i.e., in the absence of TPA), the percentage of reactive cells increases over the first week. The reactive cell percentage increased ~1.6 fold from 2-day-old to older pigs (Fig. 3A). Though the reactive cell percentage of the 14-day-old pigs was higher than that of the 2-day-olds, the intensity of the NBT reaction was greatest in a higher proportion of 2-day-old reactive cells than in reactive cells from older animals. More specifically, at all ages, the majority of reactive cells scored in the light blue (+) category (data not shown), but the percentage of reactive cells in the very intense category (+++, dark blue violet) was significantly greater ($p < 0.05$) in the cells from the 2-day-old animals than older pigs (Fig. 3B). No significant differences in intensity were detected among the 4-, 7-, and 14-day olds. Color intensity is associated with the cell's ability to produce superoxide anion; individual cell response (intensity reaction) generally decreases as a function of higher cell densities, due to a significant increase in the number of basal reactive macrophages [17]. Consistent with this interpretation, AM percentage in the BALF was higher in 4-, 7-, and 14-day-old pigs as compared to 2-day-old piglets (Fig. 2). Thus with increasing age, the percentage of cells that were reactive increased, but the degree of response of individual cells diminished: neonates possess fewer but more intensely reacting cells, while older animals had a greater number but less intensely reacting cells.

The NBT-only assay represents the basal redox metabolism state. In the presence of TPA, cells are stimulated to generate superoxide anion to their maximum ability. We found no significant

difference in the maximal values (percentage of TPA-stimulated reactive cells) across age groups. When TPA was added to the AMs (Fig. 3A), the reactive cell percentage increased at all ages examined as compared to the TPA-free assay (though the increase was not statistically significant in the 14-day-old animals). AMs thus retained a further ability to react in the event of stimulation. The increase was greatest in the AMs from the 2-day-old, which rested at a lower basal state than the AMs from the other age groups, based on the NBT-only assay. The percentage of TPA-stimulated reactive cells in the first week was not statistically significantly different from that of the second week.

3.4. Phagocytosis of opsonized particles increases with age

The percentage of phagocytic AMs in 2-day-old piglets (~34%) was significantly lower than that of older animals (~46% at 2 weeks). Phagocytic ability in response to opsonized particles increased during the first postnatal week and then appeared to plateau (Fig. 4A). Phagocytic efficiency (the number of particles internalized per cell) also showed age-dependent change. The percentage of AMs that phagocytized 1-5 beads showed a modest increase over the two week period, increasing from 33% in the 2-day-old animals to 42% in the 14-day-olds (Fig. 4B). The percentage of AMs that were highly phagocytic, i.e., that phagocytized more than five beads/cell, increased dramatically in the first week, from 0.5% on day 2 to 2.6% on day 7 (Fig. 4B). No AMs were observed to have internalized more than ten beads/cell, and at all ages, the overwhelming majority (>90%) of phagocytic cells internalized less than five beads/cell.

4. Discussion

In this paper, we describe age-related changes in BALF cellular content and AM phagocytic function that accompany the period of postnatal alveolar formation. Our major finding was that maturational changes in AM function occur very early postnatally in pigs, predominantly within the first week.

AMs were relatively less abundant in structurally immature pig lungs and characterized by a lower oxidative burst in the unstimulated state. As AM cell density in the lung increased, however, there was some evidence that individual macrophages became less efficient at superoxide anion secretion. This autoregulation is thought to be an intrinsic mechanism of macrophages associated with the protection of host tissue from the release of toxic oxygen products during the respiratory burst [17]. In rats, it has been hypothesized that high levels of cellular antioxidant activity in the AMs of neonates may contribute to the low oxidant-generating capacity during this period [18]. Interestingly, other work has shown that macrophage migration inhibitory factor, a modulator of innate immune response and redox regulated target [31], also shows age-dependent change [32]. Consistent with earlier investigations [8], we found that the phagocytosis percentage of opsonized particles was lower in 2-day-old pigs than in older pigs and there was little age-related change in the proportion of phagocytic cells after 7 days. The number of phagocytic cells that had internalized a large number (>5) of beads increased rapidly in the first week and then changed little over the second week. Lamb AMs similarly show enhancement in the first week and attain adult levels of opsonized particle phagocytosis on day eight [14].

Caution is necessary in extrapolating results from assays of phagocytic ability using opsonized beads in isolated AMs to the condition in the intact animal, as *in vitro* studies cannot fully replicate the natural lung milieu. For example, the availability of endogenous opsonins could be a limiting factor in newborns [14]. AM maturation, moreover, is likely influenced by the many changes occurring in the neonatal and postnatal lung environment [33]. For example, surfactant production and composition changes postnatally in pigs [34]. One hypothesis for the low AM activity in neonates is exhaustion from ingestion of surfactant [35]; in many species, large perinatal increases in the number of AMs is accompanied by a simultaneous

large release of surfactant, which may be involved in the maturation of AMs, and which in large quantities, may decrease AM activity [11].

Although analysis of BALF cell content is widely employed both in basic and clinical research, the use of AMs harvested from BALF raises the question of the extent to which the lavaged cells truly represent the *in vivo* condition. Macrophages recovered by lavage appear to constitute representative samples of the general AM population pool of the mouse lung [36]. Increased adherence of AMs to alveolar structures can decrease the yield of AMs in BALF, however [37], thus recovery by lavage could imply a reduction in the adherent ability of the cells, and potentially functional implications. It is difficult to compare activation in the *in vivo* and *in vitro* state, but the rate of protein synthesis in rabbit AMs suggests that lavage may result in partial activation [38]. On the other hand, in hamsters, clearance from lavaged animals was not significantly different from that of controls [39]. We do not believe that age-dependent bias in the region of the lung lavaged was a factor within the two-week timeframe of our study, given that the lungs are already quite structurally advanced at birth and the relatively large lavage volumes used.

The AM differentiation process is not well characterized [33], particularly in pigs, for which there is a paucity of suitable markers. Because CD68 expression depends on the state of cellular differentiation [40] and the antibody CD68 EBM11 clone is known to label adult pig AMs [30], we investigated the postnatal expression of this marker. At all ages examined, we found cells in the lung parenchyma displaying positive immunostaining for this marker. This CD68 expression in neonate AMs is consistent with the precocial state of pig lung maturity at birth, and with previous work showing early expression of other macrophage markers in pigs, such as the fetal expression of MAC387 [41].

Neonates can be thought of as compromised hosts and likely represent a population with heightened susceptibility to injury from inhaled particles or microbes [14]. AMs are involved in a diversity of biological functions [42], and in particular, innate immunity. The early postnatal period is unique in that the lung is still undergoing morphogenesis while the naïve neonate is experiencing its first exposure to a variety of new potential pathogens. Both changes the relative proportions of cell types and in cellular functional maturity may contribute to age-dependent improvement in AM performance [14]. Early impairment of AM function may be one factor underlying the enhanced susceptibility to pulmonary infections found in piglet neonates, as well as the neonates of other species.

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Abbreviations

AM	alveolar macrophage
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid

FITC	fluorescein isothiocyanate
FCS	fetal calf serum
NBT	nitroblue tetrazolium
PBS	phosphate buffered saline
PMN	polymorphonuclear neutrophil
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
TPA	12-0-tetradecanoylphorbol 13-acetate

Fig. 1.

Representative histological images of lung parenchyma from a postnatal series of pig lungs stained with Mallory's trichrome or immunolabeled using a macrophage marker (insets). Over the first two weeks, parenchymal airspaces become smaller due to secondary alveolar septation. Arrows indicate septal buds. *Insets:* CD68 EMB11 antibody visualized with DAB (brown) labeled alveolar macrophages (arrowheads) at all ages examined. Mayer's hematoxylin counterstain.

Fig. 2.

Differential cell count percentages from BALF of a postnatal series of piglet lungs. The percentage of AMs in the BALF increased over the first week and the percentage of neutrophils decreased. Asterisk and plus symbol indicate a statistically significant differences between the 2 day old and 4 day old ($p < 0.05$) mean percentages of AMs and PMNs respectively. Error bars represent ± 1 standard deviation. $n=3-4$ pigs per age group, counts made from 3 slides/pig.

Fig. 3.

Generation of superoxide anion through age. A) Percentage of reactive cells as estimated by the NBT reduction assay, with (gray bars) and without (black bars) TPA. The percentage of basal reactive cells (no TPA stimulation) is lower at 2 days than in older animals (*, $p < 0.05$). The percentage of reactive TPA-stimulated cells went up in all age groups, though the increase was not always statistically significant. Error bars represent ± 1 standard deviation. $n = 4-5$ pigs/age group. B) NBT reaction color intensity. The percentage of reactive cells scoring as very intense (dark blue-violet staining) was significantly higher (*) in the AMs from 2-day-old piglets than from older animals. Error bars = ± 1 standard deviation, $n = 3-4$ pigs/age group, ~ 100 cells/pig. Inset light micrographs (imaging at 100x) show representative heterogeneous response with varying degree of reaction among cells obtained from 2-day-old pigs. Note: - (no reaction), + (scattered light blue granules), ++ (increased number of granules, strong response), +++ (dark violet color, very intense).

Fig. 4.

Phagocytosis through age. A) Phagocytic ability: percentage of all cells that were phagocytic. The phagocytic percentage for the AMs of 2-day piglets is significantly lower than for all other ages (*). B) Phagocytic efficiency: percentages of all cells that internalized 0, ≤ 5 , and >5 beads/cell. Star indicates significant difference between the percentage of AMs without internalized beads at 2 days and all other ages; plus sign indicates significant difference in the percentage of AMs with ≤ 5 beads/cell at 2 days versus 14 days. Rescaled inset shows percentage of total cells that were highly phagocytic, i.e., containing >5 internalized beads. Asterisks indicate statistically significant differences in the means of the 2-day, 4-day, and 7-day AMs. Statistical significance set at $p < 0.05$. Error bars represent ± 1 standard deviation. $n = 3$ pigs/age group, cell counts represent >100 cells/plate, 3 plates/pig. Percentages represent the difference between the number of bead-associated cells at 37°C and 4°C , a temperature known to block phagocytosis.