Retinoic acid treatment partially rescues failed septation in rats and in mice

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Massaro, Gloria D., and Donald Massaro. Retinoic acid treatment partially rescues failed septation in rats and in mice. Am J Physiol Lung Cell Mol Physiol 278: L955–L960, 2000.—Pulmonary alveoli are formed in part by subdivision (septation) of the gas-exchange sacculles of the immature lung. Septation results in smaller, more numerous structures (alveoli) and is developmentally regulated in mammals including humans, rats, and mice; if it fails to occur at the appropriate time, there is no spontaneous post hoc septation nor has there been any known means of inducing septation after it has failed to occur spontaneously.

Recent work has shown that all-trans retinoic acid (RA) plays a key role in the induction of the formation of septa. For example, 1) during the period of spontaneous septation in rats, treatment with RA induces the formation of additional septa (21); 2) treatment of rats with dexamethasone (Dex) during the period of spontaneous septation inhibits septation, but concurrent treatment with RA prevents the inhibition (21); and 3) treatment with RA induces the formation of septa in adult rats with preexisting elastase-induced emphysema (22).

To further explore the conditions under which RA might induce septation, we now tested the hypothesis that treatment with all-trans RA will induce septation in a rat model of pharmacologically caused failure of septation (4, 19, 21, 32) and in mice with a genetic failure of septation (25, 37). We found in both animal models of failed septation that treatment with RA resulted in post hoc septation.

METHODS AND MATERIALS

Animals. We purchased female C57BL/6-Fbn1Tsk+/+ pa mice, female C57BL/6 mice, and timed-pregnant, specific pattern-free Sprague-Dawley rats. All were maintained in the Department of Comparative Medicine (Georgetown University, Washington, DC) on a 12:12-h light-dark cycle and were allowed food (rodent laboratory chow 5001, Ralston Purina, St. Louis, MO) and water ad libitum. Our decision to use rats of the same sex was based on the different rate of maturation of the architectural response of the lung to corticosteroids in female and male rats (20). We do not know if this applies to other hormones, e.g., RA, so we elected to use just one sex. We chose males because of the evidence that the regulation of the formation of alveoli is more complex in females than in males (24). The issues of sex differences in the timing of the response to hormones and of the regulation of alveolar formation are important, but we elected not to address either as part of this investigation. The difference in the onset of treatment in mice and rats simply represented convenient times after the cessation of spontaneous septation (2, 7).

Drugs and treatments. We injected the rats subcutaneously each day from age 4 through 13 days with 0.075 M NaCl (saline) or an equivalent volume of Dex (0.25 µg/day) in saline. This provided two initial groups of rats: saline treated and Dex treated. At age 24 days, we subdivided the rats into four treatment groups: a group designated Dil-Oil was composed of rats injected subcutaneously from age 4 through 13 days with saline (diluent (Dil)) and subsequently injected intraper-
is the mean volume of an alveolus. Vva was determined by point counting on three prints with a square-lattice test system that had test points 0.4 cm apart. More than 900 points/rat were counted, thereby attaining a relative error of between 5 and 10% (43). Alveolar surface area (Sa) was determined with point and intersection counting (43).

Statistical methods. For each parameter measured or calculated from measurements, values from individual animals from each experimental group were averaged and the SE was calculated. The Kruskal-Wallis test was used when more than two groups were compared, and the Mann-Whitney test was used to compare two populations at a time (10a). The Bonferroni method was used to adjust the significance level to the number of comparisons performed (34).

RESULTS

Rat studies. At age 37 days, rats treated with Dex from age 4 through 13 days weighed 6–12% less but had lungs that were 10% larger than rats treated with saline during the same period; treatment with RA from age 24 through age 36 days did not influence body weight or lung volume (data not shown). The volume density (Vv) of the components (alveolar air space, alveolar duct air space, alveolar wall, and conducting structures) of the gas-exchange region examined was not different among the four treatment groups (data not shown) except for the Vv of the alveolar duct air space, which was greater in Dex-RA rats (0.44 ± 0.02) than in Dex-Oil rats (0.36 ± 0.02; P = 0.018). This difference is consistent with the presence of smaller alveoli (see below) and hence of larger alveolar ducts in Dex-RA rats than in Dex-Oil rats.

Dil-RA rats had smaller, more numerous alveoli than Dil-Oil rats (Fig. 1). Dex-Oil rats had alveoli that were three times larger than those in Dil-Oil rats (Fig. 1). Furthermore, the Vv in 37-day-old Dex-Oil rats (21.2 ± 104 ± 2.2 × 104 µm3) is virtually identical to the value (19.7 ± 3.5 × 104 µm3) we (20) previously found in 2-day-old rats. Thus treatment with Dex from age 4 through 13 days completely stopped septation, and the inhibitory effect of Dex on septation was not reversed.
by the absence of treatment with Dex from day 14 through day 36. In contrast, the administration of RA to rats previously treated with Dex (Dex-RA) decreased the size and increased the Na (Fig. 1).

The frequency distribution of the volume of individual alveoli in 37-day-old rats (Fig. 2) sheds light on alveolar events. In Dil-Oil rats, only 11% of alveoli had a volume \( \geq 10 \times 10^4 \mu m^3 \) (a value that is just greater than 2 SD from the mean). The \( \bar{v}_a \) in Dil-RA rats was smaller than that in Dil-Oil rats; only 5% of alveoli in Dil-RA rats had a volume \( \geq 10 \times 10^4 \mu m^3 \). The \( \bar{v}_a \) in Dex-RA rats was shifted leftward (smaller) compared with that in Dex-Oil rats; only 43% of alveoli in DEX-RA rats were \( \geq 10 \times 10^4 \mu m^3 \), but 67% of alveoli in Dex-Oil rats were \( \geq 10 \times 10^4 \mu m^3 \). The leftward shift of the \( \bar{v}_a \) in both groups of RA-treated rats and the greater Na in RA-treated rats indicate that the larger gas-exchange structures in the Dil-Oil and Dex-Oil rats had been septated.

\[ S_a \text{ in Dil-Oil rats } [2,213 \pm 98 (SE) cm^2; n = 7] \] was lower (\( P = 0.02 \)) than the \( S_a \) in Dil-Oil rats (2,622 \pm 111 cm^2; \( n = 6 \)). Treatment with RA did not increase \( S_a \) in Dil-RA (2,817 \pm 143 cm^2; \( n = 7 \)) or Dex-RA (2,079 \pm 98 cm^2; \( n = 7 \)) rats compared with that in Dil-Oil and Dex-Oil rats, respectively.

Mouse studies. The average body mass was not different among the groups of mice (data not shown). Lung volume and body mass-specific lung volume were higher in each Tsk group than in C57BL/6 mice, but there was no difference in either parameter between Tsk-Oil and Tsk-RA mice (data not shown).

An examination of histological sections of the lung, without recourse to measurement of the volume of individual alveoli, demonstrated that the gas-exchange structures of C57BL/6 mice were much smaller than those of Tsk mice (Fig. 3). This observation was supported by measurement of the distance between alveolar walls (\( L_m \)). The \( L_m \) of C57BL/6 mice was \( 56 \pm 4 \) (SE) \( \mu m \) (\( n = 4 \)); in Tsk-Oil mice, the \( L_m \) was \( 115 \pm 8 \) \( \mu m \) (\( n = 4 \)); and in Tsk-RA mice, it was \( 108 \pm 3 \) \( \mu m \) (\( n = 5 \)); the \( P \) value between C57BL/6 and Tsk-Oil mice was 0.02, and between C57BL/6 and Tsk-RA mice, the \( P \) value was 0.014. The \( L_m \) did not differ between the Tsk groups (\( P = 0.46 \)).
The $L_m$ measurement includes alveolar duct air space and alveolar air space and, therefore, is an insensitive estimate of the size of alveoli. To determine whether treatment with RA did induce septation in Tsk mice, we measured the $v_a$ and calculated the $N_a$. The alveoli were 2.7-fold smaller and ~3.5-fold more numerous in Tsk-RA mice than in Tsk-Oil mice (Fig. 4). This difference most likely reflects septation of the large gas-exchange sac- cules as shown by the diminished frequency of large alveoli in Tsk-RA compared with that in Tsk-Oil rats (Fig. 5).

The higher number and the smaller alveoli in Tsk-RA compared with Tsk-Oil mice resulted in a greater $S_a$ in Tsk-RA mice (285 ± 11.9 cm$^2$; n = 4) than in Tsk-Oil mice (224 ± 8.5 cm$^2$; P = 0.02; n = 4). These differences were also present when $S_a$ was corrected for body mass (data not shown). In association with more numerous alveoli in Tsk-RA mice, the $v_a$ was higher in Tsk-RA than in Tsk-Oil mice; we did not detect intergroup differences in the $v_v$ of alveolar ducts, alveolar walls, or conducting structures (data not shown).

**DISCUSSION**

A "critical" period for septation. Mammals form alveoli, in part, by the developmentally regulated subdivision (septation) of the sacculles that constitute the gas-exchange region of the architecturally immature lung (2–7, 13, 15, 16, 18–21). Treatment of rats with Dex during the period in which they would normally septate (3–7) prevents septation (4, 19, 21, 32), and spontaneous post hoc septation does not occur after treatment with Dex is ended; i.e., there is a developmentally regulated critical period in which septation must occur (4, 19, 32). In addition to experimental inhibition of septation, there may be a genetic failure of septation (25) as occurs in Tsk mice (11) in which a tandem duplication within the fibrillin-1 gene is associated with the Tsk mutation (35). Finally, septation may be im- paired in prematurely born human infants (13, 16, 36).

RA and the formation of alveoli. The molecular signals responsible for the eruption of alveolar septa, for the metabolically related spacing of their eruptions (39), and for the developmental regulation of the timing of eruptions (2–7, 18–21) are poorly understood. However, in the rat, several lines of evidence have suggested that retinoids might play a key regulatory role in septation. These observations are that 1) fibroblasts rich in vitamin A (retinol) storage granules (28) occupy a large fraction of the alveolar wall during the period of septation but diminish after septation has ended (17, 42); 2) during the period of septation, the concentration in the lung of cellular retinoic acid binding protein I peaks and that of cellular retinol binding protein I (CRBP-I), a key protein in the synthesis of RA (26), is high (29); 3) treatment with Dex, which prevents septation (4, 19, 21, 32), diminishes the concentration of CRBP-I mRNA (31); 4) treatment with RA upregu- lates the concentration of CRBP-I mRNA (31); 5) thy-roid hormone induces more rapid septation (18); and 6)
thyroid hormone and RA can induce gene expression through a common response element (41).

More direct evidence that RA can regulate septation is shown by the following: 1) daily treatment with RA of otherwise untreated rats from age 4 through 13 days, which is the period of spontaneous septation (6, 7), causes a 50% increase in the N a formed without an increase in V L (21); 2) daily treatment of rats with RA from age 4 through 13 days prevents the inhibition of alveolar formation and the low body mass-specific S a caused by treatment with Dex during the same period (21); 3) RA induces septation in explants of fetal mouse lungs (8); 4) RA treatment of adult rats, previously made emphysematous by the intratracheal instillation of elastase, causes the induction of septation (22) and in rats abrogates key features of human (40) and experimentally (14) emphysema; and 5) treatment of prematurely delivered sheep with retinol increases the N a (1).

Individual alveoli, N a, S a, and the regulation of alveolar formation. Treatment with RA increased N a in rats previously treated with RA and in RA-treated Tsk mice but not in the Tsk mice that did treatment with RA increase the S a. The reason for this difference is unclear to us, but certain possibilities are worth discussing. If alveolar shape is the same in both groups, S a is related to volume to the power 2/3. Therefore, a change in volume is accompanied by a change in S a that is only ~60% as large. A much bigger difference in the volume of an average alveolus was produced by RA treatment in Tsk mice (2.7-fold) than in rats treated with RA after prior treatment with Dex (1.6-fold). These factors may have contributed to the detection of a change in Tsk mice but not in the rats.

Viewed differently, the absence of an intergroup difference in S a, together with differences in alveolar size and number, raises interesting issues about the regulation of the eruption of septa and the eventual size of alveoli. It is unclear why, in the face of diminished S a per body mass in Dex-Oil rats and increased septation in Dex-RA rats, there is no return toward the values in Dil-Oil rats of the body mass-specific S a in Dex-RA rats. An explanation of these findings is that RA induces the eruption of septa and regulates the distance between septa but does not determine the ultimate size of alveoli (length of septa) and thereby of S a. The evidence from the present work, our prior work (21, 22), and the work of others (1, 8) makes it clear that exogenous RA induces the eruption of septa. The presence of smaller, more numerous alveoli in neonatal retinoic acid receptor-β knockout mice than in wild-type founder mice, without an intergroup difference in V L or S a (23), indicates that endogenous retinoids play a role in the eruption of septa but not in determining V L or S a. Therefore, we propose that retinoids regulate the eruption of septa and their spacing, whereas another regulator(s) determines the S a. If this is the case, there must be feedback inhibition of septal elongation to prevent increased S a in the face of more septa when additional S a is not needed.

Clinical relevance. There are three major lung diseases in which there may be too few alveoli for adequate gas exchange: two, diffuse interstitial fibrosis and pulmonary emphysema, are characterized by alveolar destruction, and the third, bronchopulmonary dysplasia, mainly reflects a failure to sequestate but also has a component of alveolar destruction. In none of these diseases is there a means, short of lung transplantation, to remediate the insufficient N a. However, now in three species; in premature, immature, and adult animals; and in three animal models with a low N a [elastase-induced emphysema (14), genetic failure of septation in Tsk mice (25), and Dex-produced failure of septation (3, 19, 21, 32)], treatment with RA induces alveolus formation. These findings offer the possibility of a similar remedial action in humans.

We are deeply grateful to Dr. Luis Cruz-Orive for reviewing an earlier version of the paper. We thank J. Oyce for technical help and Drs. Linda B. Clerch and Wai-Yee Chan for critically reading an early version of this manuscript.

This work was supported by National Heart, Lung, and Blood Institute Grants HL-37666, HL-20366, HL-59432, and HL-60115. G. D. Massaro and D. Massaro are Senior Fellows of the Lovelace Respiratory Research Institute (Albuquerque, NM). D. Massaro is Cohen Professor of Pulmonary Research, Georgetown University (Washington, DC).

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Received 24 September 1999; accepted in final form 10 December 1999.

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