Retinoic acid in alveolar development, maintenance and regeneration

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Recent data suggest that exogenous retinoic acid (RA), the biologically active derivative of vitamin A, can induce alveolar regeneration in a rat model of experimental emphysema. Here, we describe a mouse model of disrupted alveolar development using dexamethasone administered postnatally. We show that the effects of dexamethasone are concentration dependent, dose dependent, long lasting and result in a severe loss of alveolar surface area. When RA is administered to these animals as adults, lung architecture and the surface area per unit of body weight are completely restored to normal. This remarkable effect may be because RA is required during normal alveolar development and administering RA re-awakens gene cascades used during development. We provide evidence that RA is required during alveologenesis in the mouse by showing that the levels of the retinoid binding proteins, the RA receptors and two RA synthesizing enzymes peak postnatally. Furthermore, an inhibitor of RA synthesis, disulphiram, disrupts alveologenesis. We also show that RA is required throughout life for the maintenance of lung alveoli because when rats are deprived of dietary retinol they lose alveoli and show the features of emphysema. Alveolar regeneration with RA may therefore be an important novel therapeutic approach to the treatment of respiratory diseases characterized by a reduced gas-exchanging surface area such as bronchopulmonary dysplasia and emphysema for which there are currently no treatments.

Keywords: retinoic acid; lung regeneration; alveologenesis; vitamin A; emphysema; mouse

1. INTRODUCTION

The recent discovery of the presence of stem cells in the adult mammalian body and the generation of embryonic stem cells has raised the possibility of using these cells to replace those lost by disease, damage or old age and thus serve to re-seed and regenerate adult organs. This is a marvellous concept for medicine, but practically, enormous problems lie ahead, including the difficulty in establishing human embryonic stem cell lines, obtaining enough adult stem cells, knowing how to differentiate them into discrete, differentiated populations, and potential immunological rejection after grafting into a recipient. Would not all these problems be negated if the mammal could be induced to regenerate its own individual organs?

Organ regeneration is rather randomly distributed throughout the animal kingdom, but the champion regenerators must be the Urodeles, who can regenerate the spinal cord, limbs, tails, lower jaws, heart muscle, gills, retina, lens and small intestine (Goss 1969). Organ regeneration in mammals is usually considered to be present in only the skin and liver, but imagine what medical possibilities would present themselves if cardiac muscle could be induced to regenerate, if the spinal cord could repair itself, if islets of Langerhan could regenerate, to name but three.

In the work described herein we demonstrate that pre-

viously unsuspected regenerative capacity is present in mammalian lungs and that it can be awakened with the application of a low molecular mass lipophilic molecule called RA. As this molecule is rapidly diffusing, freely enters cells and alters the pattern of gene activity by switching on and off transcription, it is the perfect pharmaceutical compound.

2. RETINOIC ACID AND ITS SIGNALLING PATHWAY

RA is an important signalling molecule found in the embryonic and adult vertebrate, which is derived from vitamin A. It is of low molecular mass (300 Da) and is lipophilic. In the adult, vitamin A is obtained from the diet in the form of retinyl esters present in animal meat, or β -carotene present in plants. Cells of the embryo or adult that require RA obtain it from the blood system, where it circulates as retinol bound to retinol binding protein. In the cytoplasm of cells, retinol is bound to a cytoplasmic protein, CRBP, which facilitates its enzymatic conversion to RA (Napoli 1999). The sequestered retinol is first converted to retinal by the retinol or alcohol dehydrogenases and then to RA by the retinal dehydrogenases (Duester 2000). RA is bound in the cytoplasm by CRABP, which may assist in the functioning of RA in the nucleus (Delva *et al.* 1999; Dong *et al.* 1999). Having functioned in the control of gene transcription, RA is catabolized by a family of cytochrome P450 enzymes called CYP26 to supposedly inactive products such as 4-oxo-RA, 4-OH-RA, 18-OH-RA and 5,18-epoxy-RA (White *et al.* 1996; Fujii *et al.* 1997; Abu-Abed *et al.* 1998) and finally excreted. There

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Figure 1. The effect of RA on limb regeneration in the axolotl. (a) A control limb amputated through the mid-forearm (black line) which regenerated a perfect copy of what was removed, i.e. the ends of the radius and ulna, the carpals and digits. (b) A limb from an RA-treated animal which was also amputated through the mid-forearm level (black line) but which regenerated a complete limb from the amputation plane beginning with the humerus rather than just the lower arm and hand.

are two isomers of RA, *all-trans*-RA and *9-cis*-RA, which act via different receptors (see next paragraph), but whether they have separate enzymatic pathways from *all-trans*-retinol and *9-cis*-retinol or isomerization takes place as a last step is not really known.

Once RA has been synthesized in the cytoplasm it enters the nucleus and establishes or changes the pattern of gene activity by binding to ligand-activated nuclear transcription factors. There are two classes of these transcription factors, the RARs and RXRs. In human, rat and mouse there are three RARs, α , β and γ , each of which has multiple isoforms (Kastner *et al.* 1994), and three RXRs, α , β and γ (Kliwer *et al.* 1994), again each having several isoforms. The ligand for the RXRs is *9-cis*-RA whereas the RARs bind both *9-cis*-RA and *all-trans*-RA. The RARs and RXRs act as heterodimers (e.g. RAR α /RXR β) and recognize consensus sequences known as RAREs in the upstream promoter sequences of RA-responsive genes.

3. RETINOIC ACID AS A REGENERATION-INDUCING MOLECULE

RA first came to prominence as a molecule involved in regeneration when it was shown that it causes 'super-regeneration' in those organisms that can already regenerate, namely the Urodele amphibians (Maden 1982). Normally, following amputation of an axolotl or newt limb anywhere along its length a perfect copy of what was lost will be regenerated in a few weeks (figure 1a). But if RA is applied either locally in the limb, by intraperitoneal injection or by simply adding to the water in which the animals live, then more limb structures than were removed are replaced (figure 1b). In fact, there is a concentration-dependent increase in the amount of extra tissue regenerated. For example, after amputating through the middle

of the forearm a normal limb will replace the lower arm and hand (figure 1a). At a medium dose of RA two forearms and a hand are regenerated and at a high dose of RA an extra complete limb is regenerated from the lower arm amputation plane (figure 1b). In frog tadpoles this super-regeneration effect of RA leads to the regeneration of complete pairs of hindlimbs from an amputation plane through the foot instead of simply regrowing the foot as controls do (Maden 1983).

Does this property of RA of inducing regeneration have any relevance to mammals? Amazingly, it seems the answer is yes because it has been shown that RA can induce the regeneration of lung alveoli.

4. ALVEOLAR REGENERATION IN THE ADULT LUNG

It was recently discovered that RA can induce the regeneration of lung alveoli in the experimentally damaged adult rat lung (Massaro & Massaro 1997). The experimental damage induced was the instillation of elastase into the trachea, which destroys the alveoli, resulting in a vastly reduced gas-exchanging surface area and this was developed as a model for the human disease of emphysema. This effect of RA was also seen in the tight-skin mutant mouse, which has a genetic failure of alveolar development and in postnatal rats treated with dexamethasone (Massaro & Massaro 2000). Subsequent studies have, however, shown only a partial rescue of alveolar surface area in elastase-treated rats (Belloni *et al.* 2000; Tepper *et al.* 2000), but others have shown no effect of RA on lung function (Srinivasan *et al.* 2000).

We have developed a dexamethasone-treated mouse model for studies of regeneration and have specifically chosen to characterize this phenomenon in the mouse because of the availability of transgenic technology for future experiments.

5. NORMAL ALVEOGENESIS

The relative timing of the alveologenesis is species dependent. Mammals that have fur and are mobile at birth, precocial animals such as sheep, deer and guinea-pigs, are born with functional alveoli whereas mammals that are entirely dependent on their mothers, altricial animals such as rats, mice and humans, are born with primitive gas-exchanging structures that undergo alveologenesis largely as a postnatal event. This seems to be an evolutionary phenomenon reflecting the different requirements for gas exchange in the lungs of different species at birth (Massaro & Massaro 1996).

Morphometric studies where the number, size and gas-exchanging surface area of alveoli can be calculated suggest that the majority of alveoli are formed between day 4 and day 14 in the rat and mouse (Burri *et al.* 1974; Amy *et al.* 1997) and probably up to 18 months in humans (Zeltner *et al.* 1987). During these first two weeks after birth in rats and mice, from the thick walls of the primitive air sacs which have a double capillary network, erupt alveolar septa, which divide the airspace into smaller alveoli. The mature alveolar walls have a single capillary structure and the reduction in capillaries and thinning of the alveolar walls are attained by apoptosis within each of

Figure 2. The development of alveoli in the postnatal mouse. (a–c) Haematoxylin and eosin stained sections of lungs, all at the same magnification. (a) A postnatal day 4 (P4) lung showing thick walls and large airspaces. (b) A P9 lung showing thinner walls and alveolar septa growing out (arrowheads) and cutting up the large airspaces into smaller ones. (c) A P15 lung showing the completion of alveologenesis with small airspaces and thin walls. (d) L_m data of lungs from P14 to adulthood showing that the L_m (a measure of the alveolar diameter) does not change. Each time point represents 30 measurements from three animals with standard errors marked.

the populations of cells making up the alveolar walls (Schittny *et al.* 1998; Bruce *et al.* 1999). As a result of dissecting up the air sacs the alveolar diameter decreases. Figure 2a shows a postnatal day 4 mouse lung with thick walls and large alveoli. Figure 2b, at the same magnification as figure 2a, shows a postnatal day 9 lung with alveolar septa growing out (arrowheads) to cut large alveoli up into smaller units. Figure 2c shows a postnatal day 15 lung with many small alveoli with thin walls. After these first two weeks, the alveolar diameter, which is known as the L_m , does not change and remains stable throughout the adult period. Figure 2c shows data that demonstrate this phenomenon, the consistent L_m value after postnatal day 14 up to and throughout adulthood.

The cells that make up the walls of the developing alveoli show interesting and important features. The type I cell is the fully differentiated squamous epithelial cell with thin attenuated cytoplasm forming the barrier between the air and the capillary. There is little metabolic activity in this cell, whose function is to provide an intact surface of minimal thickness readily permeable to gases. The type II cell is thought to be the progenitor for the type I cell. Type II cells account for *ca.* 50% of the alveolar epithelial cell numbers, but only 7% of the surface area (Crapo *et al.* 1983). They have a characteristic morphological appearance of a large rounded cell, are found in the corners of the alveoli and are in close proximity to connective tissue cells lying beneath them. As well as functioning as a potential stem cell the other main function of

the type II cell is in the production of pulmonary surfactants. The connective tissue cells lying beneath are known as lipofibroblasts.

There are two types of fibroblastic, connective tissue cells or interstitial cells: lipid-laden and non lipid-laden. The lipid-laden fibroblasts participate in the synthesis of structural protein such as collagen and elastin, they have an accessory role to the type II cell in the production of surfactant and are the pulmonary storage cell for retinoids (Okabe *et al.* 1984). Signalling between the type II cell and the lipofibroblast might be crucial for normal alveologenesis, because Burri *et al.* (1974) noted that there was an accumulation of lipid-laden fibroblasts adjacent to the type II cell at septal junctions.

Elastin is the major structural component of the alveolus. Elastin synthesis begins at midgestation, peaks near birth in the early postnatal period then drops sharply and is nearly totally repressed in the adult (Powell & Whitney 1980; Dubick *et al.* 1981; Noguchi & Samaha 1991). Mice that are null mutants for the elastin gene have defective lung development immediately after birth (Wendell *et al.* 2000).

6. DEXAMETHASONE EFFECTS ON THE LUNG

It is known that exogenous administration of dexamethasone prevents the normal formation of alveoli in the rat (Massaro *et al.* 1985; Tschanz *et al.* 1995). It accelerates alveolar wall thinning and decreases cell number by

inhibiting replication, but the precise mechanism by which dexamethasone acts is not understood.

We have developed a dexamethasone-treated mouse model for our studies of alveolar regeneration. When dexamethasone is administered during the first two post-natal weeks in mice (days P2–P14), alveolar development is inhibited. There is a striking loss of alveoli and an increase in alveolar diameter (L_m). We first determined the effect of increasing the dose of dexamethasone (0.05, 0.2 and 0.4 mg kg⁻¹ body weight) administered. At the end of the dosing protocol the mice were left undisturbed and sacrificed at P30. Figure 3*a–d* shows the morphology of the lungs after increasing doses and it is clear that the medium and high doses have a drastic effect on alveolar number and size. In figure 3*e* the L_m measurements are assembled and they confirm that there was a significant difference between control and medium- and high-dose groups and no significant difference between control and the low-dose group. Thus, there is a dose-dependent effect of dexamethasone on alveologenesis.

We next determined the effect of increasing the number of doses of dexamethasone. The high dose was selected and administered for 3, 5 or 10 days from P2. As in the previous paragraph, the mice were killed at P30. Figure 3*f* shows the L_m measurements of the lungs after increasing number of doses. There is no significant difference between controls and the 3 days of dexamethasone, whereas dosing for 5 and 10 days results in a significantly increased L_m at P30. Thus, from these data we settled upon the high dose of dexamethasone (0.4 mg kg⁻¹) delivered for 10 days over the P2–P14 period.

As we were trying to induce regeneration with subsequent treatment with RA it was important to establish that every animal that was treated would be deleteriously affected by dexamethasone. If one or two animals in every batch were unaffected then a spurious regeneration result would be obtained. To demonstrate the uniformity of the dexamethasone effect, a group of 13 animals was treated and a parallel control group treated with PBS. The L_m values were measured on day 30, and figure 4*a* shows that all 13 of the experimental animals had a significantly greater L_m than any of the controls. As in the developmental series described earlier, the controls had L_m values that varied around 60 (average of 63.3). The dexamethasone-treated L_m values varied from 80 to 110 (average of 91.9).

We also had to establish that the effect of dexamethasone was permanent and that the treated animal would not spontaneously regenerate. Accordingly, a group of animals ($n = 4$) were dexamethasone treated and left to grow long into adulthood. After 150 days they were killed and the L_m values measured, whereupon the differences had been maintained. Figure 4*b* shows the lung from a 150-day control animal and figure 4*c* a 150-day dexamethasone-treated animal with massively increased alveolar diameters and loss of gas-exchanging surface area. This confirms that the dexamethasone effect lasts for at least five months.

7. INDUCTION OF REGENERATION

We could now use this model system for studying regeneration. Thus, a group of animals were dexamethasone treated postnatally with the dosing protocol

Figure 3. (*a–d*) Haematoxylin- and eosin-stained sections of P30 mouse lungs at the same magnification to show the effect of an increasing dose of dexamethasone on alveolar dimensions. (*a*) Control lung showing normal alveoli. (*b*) At a dose of 0.05 mg kg⁻¹ dexamethasone very little difference can be noted. (*c*) A dose of 0.2 mg kg⁻¹ results in obviously enlarged airspaces owing to the partial failure of alveolar septation. (*d*) A dose of 0.4 mg kg⁻¹ gives uniformly enlarged airspaces. (*e*) L_m values of lungs treated with various doses of dexamethasone. Each bar represents groups of 6–10 mice with standard errors marked. The medium and high-dose L_m values are significantly different from the control (*) ($p < 0.02$). (*f*) L_m values of lungs treated with increasing numbers of doses of 0.4 mg kg⁻¹ dexamethasone. Each bar represents groups of 4–6 mice with standard errors marked. The five- and 10-dose L_m values are significantly different from the control (*) ($p < 0.001$).

established above and left to P30. They were then injected intraperitoneally with RA dissolved in DMSO and peanut oil at a dose of 2 mg kg⁻¹ daily for 10 days. Two control groups were used: one that had not received dexamethasone, only PBS injections postnatally and received DMSO/oil (these should have normal alveoli), and another that had received dexamethasone postnatally but only then received DMSO/oil and no RA (these should

Figure 4. (a) Experiment to ensure that every dexamethasone-treated animal is affected. A total of 13 mice were used as controls and on the left of the graph their L_m values are recorded. The average of this group is 63.3. On the right of the graph are 13 dexamethasone-treated animals. Every one of these animals has a higher L_m value than any of the controls and the average L_m value for this group is 91.9. (b,c) Haematoxylin- and eosin-stained sections at the same magnification of (b) a 150-day control mouse and (c) a 150-day dexamethasone-treated mouse, to demonstrate that the loss of alveoli induced during P2–P14 remains for a considerable length of time and that alveoli do not spontaneously regenerate.

remain damaged). Each group ($n=7-8$) was killed at day 90.

Figure 5a shows the typical structure of a control lung that did not receive dexamethasone or RA and figure 5b shows a typical dexamethasone-treated lung with disrupted alveolar architecture and enlarged airspaces. Figure 5c shows the complete restoration of alveolar architecture to a dexamethasone-treated animal that was subsequently given RA. The mean chord length data obtained from this histology are shown in figure 5d, where it is clear that RA has almost fully restored L_m . Further morphometric analyses were performed knowing the lung volumes to determine the gas-exchanging surface area and then this value was normalized to the body weight of the animals to eliminate the effect of body weight on alveolar number. Figure 5e shows that when these analyses are performed, RA fully restores the gas-exchanging surface area of dexamethasone-treated animals.

8. THE RELATIONSHIP BETWEEN REGENERATION AND DEVELOPMENT

It is interesting to consider why RA might have such an effect on an adult tissue as it is primarily known for its important role as a signalling molecule in several developing systems (Maden & Pizzey 1997; Ross *et al.* 2000; Maden 2002). One concept common in regenerative biology is that regeneration re-awakens the gene

pathways used during development rather than having evolved entirely separate pathways unique to regeneration. Developing and regenerating systems in which RA is already known to be involved, such as the limb, provide excellent examples of this principle. Thus, RA is present in the developing limb bud (Thaller & Eichele 1987, 1990) and without it development ceases (Helms *et al.* 1996; Stratford *et al.* 1996; Lu *et al.* 1997; Maden 1998) and RA is present in the regenerating limb blastema (Brookes 1992; Scadding & Maden 1994; Viviano *et al.* 1995) and without it regeneration ceases (Maden 1998). Presumably, then, the same RA-induced gene pathways are involved both in the developing and regenerating limb.

This may be the reason why the alveoli of the adult mammal can regenerate when RA is supplied because it used RA during alveologenesis in the first place and RA administration re-awakens those developmental gene cascades. It is thus interesting to consider whether RA and its signalling components are present during alveologenesis.

9. RETINOIC ACID AND ALVEOGENESIS

Retinoids have indeed been implicated in alveologenesis. The retinoid content of the lung changes significantly around and after birth with a decrease of storage forms and an increase in active forms such as retinol and RA (Geevarghese & Chytil 1994; McGowan *et al.* 1995). The RA-synthesizing enzymes, receptors and cytoplasmic binding proteins are present (Ong & Chytil 1976; McGowan *et al.* 1995; Whitney *et al.* 1999; Hind *et al.* 2002a,b) and mice made mutant for the $RAR\gamma$ gene fail to form alveoli correctly (McGowan *et al.* 2000). Surprisingly, mice made mutant for the $RAR\beta$ gene form too many alveoli (Massaro *et al.* 2000) suggesting that RA, acting through the RARs, can be both a positive and a negative regulator of alveologenesis.

We have examined postnatal mouse lungs for the components of the RA signalling pathway and determined how they change over the first two postnatal weeks.

(a) The binding proteins

By RT-PCR we have shown that both retinol-binding proteins CRBP I and CRBP II are present during alveologenesis (Hind *et al.* 2002a). CRBP I peaks at P9 and CRBP II peaks at P4. By immunocytochemistry CRBP I is present in the alveolar walls and not in the bronchial epithelium (figure 6a). It is not expressed by every cell but seems to be associated with cells with large vacuoles that resemble the lipid-laden fibroblasts (arrows in figure 6a). As these cells are the storage site for retinoids in the lung this makes sense. Out of the two RA-binding proteins CRBP I and CRBP II, we have only been able to detect CRBP I by RT-PCR and so we presume that CRBP II is not present during alveologenesis (Hind *et al.* 2002a). CRBP I peaks at P9 and by immunocytochemistry can be seen in the alveolar walls and not in the bronchial epithelium (figure 6b). CRBP I is particularly highly expressed in the alveolar septa which on day 9 can be seen to be dividing up the large airspaces (figure 6b).

(b) The RA-synthesizing enzymes

We have detected both RALDH 1 and RALDH 2 by RT-PCR during alveologenesis. RALDH 1 peaks at P4

Figure 5. (a–c) Haematoxylin- and eosin-stained sections of control and regenerated lungs. (a) Untreated lung showing typical small alveoli. (b) Dexamethasone-treated lung showing massive loss of alveoli. (c) RA-treated dexamethasone lung showing a complete restoration of alveolar architecture. (d) The L_m data for the three groups showing that RA restores the L_m , but not completely. (e) The morphometric data when differing body weights are taken into account show that RA does indeed fully restore lung architecture.

whereas RALDH 2 is highest before birth and expression levels slowly decline during alveologenesis (Hind *et al.* 2002b). Using immunocytochemistry, interesting spatial differences in these two enzymes are apparent. RALDH 1 is expressed in the bronchial epithelium and the alveolar walls, in the latter in large cells at septal junctions that, as in the case of CRBP I, resemble lipid-laden fibroblasts (figure 6c). Alternatively, RALDH 2 is not expressed in the alveolar walls but in the bronchial epithelium and the pleural mesothelial cells around the outside of the lung (figure 6d). Interestingly these differences correlate with differing patterns of cell proliferation that can be detected during alveologenesis, suggesting that each enzyme may have a distinct role and may perhaps synthesize distinct retinoids (Hind *et al.* 2002b).

(c) *The RARs and their isoforms*

Owing to multiple promoter usage and differential splicing there are potentially seven isoforms of the RAR α gene, four of the RAR β gene and seven of the RAR γ gene (Leid *et al.* 1992). Considerable complexity could thus be generated. Using specific primers for each of these 18 isoforms we have identified which are present during alveologenesis (Hind *et al.* 2002a) and the picture that emerges is a lot simpler. Only one RAR α isoform is present, RAR α 1; two RAR β isoforms are present, RAR β 2 and RAR β 4; and only one RAR γ isoform, RAR γ 2. Each of these isoforms is significantly upregulated at P4 (figure 6e–h) and by *in situ* hybridization they are distributed widely, being present in the bronchial epithelium, bronchial and vascular smooth muscle, pleura and alveolar walls.

10. THE INHIBITION OF RETINOIC ACID SYNTHESIS DURING ALVEOGENESIS

We have also detected RA endogenously in the post-natal lung (Hind *et al.* 2002b), as others have

(Geevarghese & Chytil 1994), which is presumably synthesized by both RALDH 1 and RALDH 2. To determine whether the presence of RA is crucial to alveologenesis we have administered an inhibitor of the aldehyde dehydrogenases, disulphiram (Vallari & Pietruszko 1982) to post-natal mice. Treating the animals daily with a dose of 2 mg kg⁻¹ disulphiram from P2 to P14 and sampling the lungs at P23 resulted in a significant increase in L_m compared with controls (figure 7a) and the histological analysis showed larger airspaces with thicker alveolar walls (figure 7b,c). Although these results were positive they must be treated with caution. Despite our demonstration that disulphiram does indeed inhibit the synthesis of RA (Stratford *et al.* 1996) there are several reports of other enzymes that are also inhibited, suggesting the lack of specificity of this compound. Future experiments to provide a more definitive answer to this question will be conducted using RAR pan-antagonists.

11. RETINOIC ACID AND ALVEOLAR MAINTENANCE IN THE ADULT

If RA is required for the development of alveoli and RA can induce the regeneration of damaged alveoli, what role could it play in the maintenance of the differentiated state in the adult? We know that the lung contains high levels of retinoids owing to their presence in the lipid-laden fibroblasts and it is therefore a pertinent question to ask what would happen to the lung in the absence of retinoids.

One of the original observations of retinoid depletion was that there were characteristic changes in the epithelium of the conducting airways (Wolbach & Howe 1925). The proximal airway cells lose their characteristic phenotype of stratified columnar epithelium with goblet cells and undergo metaplastic transformation into keratinizing squamous epithelium, but the effects of retinoid depletion on the alveolar epithelium have hardly been

Figure 6. (a–d) Immunocytochemistry on sections of postnatal lungs during the period of alveologenesis to show the distribution of the binding proteins and the enzymes. (a) P15 lung showing CRBP I protein present in the alveolar walls in cells with large vacuoles which resemble lipid-laden fibroblasts (arrows). (b) P9 lung showing CRBP I present particularly in the alveolar septa (arrows). (c) P15 lung showing RALDH 1 protein in the alveolar walls in cells with large vacuoles that resemble lipid-laden fibroblasts. (d) P1 lung showing RALDH 2 protein only in the mesothelial cells of the pleural lining of the lung and not in the alveolar walls. (e–h) RT-PCR data showing relative expression levels of the four RARs that are present during alveologenesis. (e) RAR α 1 peaks at P4. (f) RAR β 2 peaks at P4 and P9. (g) RAR β 4 peaks at P4. (h) RAR γ 2 peaks at P4.

Figure 7. The effect of disulphiram on lung architecture. (a) L_m values of control and disulphiram-treated animals at P23 ($n = 6-7$). (b,c) Haematoxylin- and eosin-stained sections at the same magnification of P23 lungs showing the loss of alveoli and alveolar damage in the disulphiram-treated cases (c).

studied. To our knowledge, only one recent report states that vitamin A deficiency results in airspace enlargement (Baybutt *et al.* 2000). We have therefore investigated this aspect in greater detail and turned to rats for this VAD experiment; these are the classical organisms for metabolic studies because it seems that mice are particularly resistant to nutritional vitamin A depletion.

A group of rats ($n = 5$) was maintained on a vitamin A-free diet from 12 weeks of age, at which time alveolar formation is complete (Burri *et al.* 1974), to 1 year when they were killed. In all other aspects including calorie intake, other vitamins and trace elements, the diet was normal. Retinoid deficiency was confirmed with HPLC analysis of blood and liver. There were obvious histological differences between control and VAD lungs (figure 8a,b). The VAD lungs appeared to have fewer larger airspaces, which was a diffuse effect, observed throughout the lung. The alveolar walls appeared thinner with more alveolar breaks, though this has not been quantified. Interestingly a peri-bronchial inflammatory cell infiltrate was noticed on many samples of VAD lungs. Morphometric analysis of L_m values confirmed the histological picture that L_m was increased after vitamin A deficiency (figure 8c).

12. CONCLUSIONS

The annual cost of injured or failing human tissues and organs is a major global healthcare expenditure running into billions of pounds per year. Over the past 50 years there has been remarkable progress in restoring both structure and function in many dysfunctional tissues through the use of mechanical devices and organ transplantation, and a new wave of success using stem cell transplantation is now anticipated. Perhaps successes in inducing organ regeneration in humans will also be possible in the future because we have described here how RA

Figure 8. The effect of long-term VAD on lung architecture. (a,b) Haematoxylin- and eosin-stained sections at the same magnification of 1-year-old adult rats. In (a) the control rat shows a typical appearance whereas in (b) there is a loss of alveoli with large airspaces caused by the VAD. (c) The L_m values show the increased average alveolar diameter associated with VAD.

can induce alveolar regeneration in the damaged mouse lung, a phenomenon that also occurs in rats (Massaro & Massaro 1997).

We assume, and will be testing this in future experiments, that the source of regenerated cells is from within the lung itself and not from bone marrow-derived multipotent stem cells (Kotton *et al.* 2001; Krause *et al.* 2001). The lung contains its own supply of stem cells, the type II pneumocytes. These are large, relatively undifferentiated cells that are often seen proliferating and are known to possess the RA signal transduction components (Naltner *et al.* 2000).

If this result translates to humans and can be used as a treatment for emphysema then it would have a dramatic effect on the world's health. Emphysema is characterized by airway destruction distal to the terminal bronchioles, gradual loss of lung recoil, decreased surface area and impaired gas exchange (Thurlbeck & Muller 1994). The most common cause of emphysema is cigarette smoking. Stopping smoking slows the rate of damage, but the damaged alveolar structures do not repair and lung function is not regained (Pauwels *et al.* 2001). Currently, there are no effective treatments for emphysema save supplemental oxygen and lung transplantation, and the outlook for affected patients is bleak (Barnes 2000). Emphysema and chronic obstructive bronchitis together comprise the disease known as COPD. COPD prevalence and mortality are on the increase and the World Health Organization predicts that by 2020 COPD will rise from its current ranking of the sixth most common cause of death to third place (Murray & Lopez 1996).

We can only hope that this technique is applicable to the human lung and in support of this idea we show that the probable reason why RA has such an effect on the adult lung is that RA is required for the development of alveoli in the first place. All of the RA signal transduction machinery is present postnatally in the lung as the alveoli

form, and disrupting the synthesis of RA disrupts alveologenesis. Therefore we suggest that RA re-awakens the gene pathways that the alveolar cells used in the first place to induce regeneration, a common concept in regenerative biology.

We also approached the question of whether the adult lung requires RA for its maintenance by depriving rats of dietary vitamin A. The result was the characteristic lung structure of emphysema, as has been seen before (Baybutt *et al.* 2000), suggesting that endogenous RA is essential for ongoing alveolar repair. Interestingly, in rats, cigarette smoke induces vitamin A depletion (Li *et al.* 2003) thereby linking the known human cause of emphysema with the role of RA in alveolar maintenance. As the alveolar structure is so fragile it is likely that the distal adult lung is a dynamic environment with variable low-grade alveolar destruction and repair going on continuously. But whether this is a direct or indirect mechanism of action of RA is not known. Two indirect mechanisms seem possible. One is that it acts via the immune system, which is known to be suppressed under conditions of vitamin A deficiency (Blomhoff & Smeland 1994) and predisposes the human population to infections (West 1994). Vitamin A supplementation reduces mortality in humans from many infectious diseases endemic in the developing world. The presence of peri-bronchial inflammatory cells observed in the VAD rats might be providing a clue and suggest that these animals were predisposed to chronic respiratory infection. Interestingly, chronic infection and inflammation are associated with the development of emphysema and COPD in both animal models and human disease.

Another far less direct mechanism may be via starvation, which is known to induce the histological appearance of emphysema and was noted in uninfected lung specimens by physicians performing post-mortem examinations in the Warsaw ghetto (Winick 1979). The same is true in animal models (Kerr *et al.* 1985; Karlinsky *et al.* 1986; Massaro *et al.* 2002), but we were careful to ensure that the calorific, trace element and other vitamin contents of the normal and vitamin A-deficient diets were equivalent, so it is unlikely that this is the explanation.

We can conclude, therefore, that RA is required for the development, maintenance and induction of regeneration of the alveolus. The latter may suggest that there is some hope for the currently incurable disease of emphysema, which, as a component of COPD, is rapidly rising in incidence throughout the world.

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GLOSSARY

- COPD: chronic obstructive pulmonary disease
 CRABP: cellular retinoic acid binding protein
 CRBP: cellular retinol binding protein
 DMSO: dimethylsulphoxide
 L_m : mean chord length
 RA: retinoic acid
 RAR: retinoic acid receptor
 RARE: retinoic acid response element
 RXR: retinoid X receptor
 VAD: vitamin A deprivation