Relationship of epithelial growth to mitotic rate in mouse embryonic lung developing in vitro

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INTRODUCTION

In an earlier paper (Alescio, 1965) we studied the in vitro growth rate of the epithelial tree of mouse embryonic lung, using as a criterion for growth the increase of the epithelial surface, due to budding activity, as measured on the living cultures.

We showed that X-irradiation brings about a dose-dependent growth inhibition (Alescio, 1966). Two experimental situations able to increase the growth rate (budding activity) of the epithelial tree in a highly reproducible way were identified: (1) reduction of the total mass of the rudiment, and (2) increase of the amount of mesenchyme relative to epithelium (Colombo Piperno, 1966; Alescio & Colombo Piperno, 1967).

The results led us to conclude that under the reported conditions of growth in vitro: (1) the pulmonary rudiments are able to undergo some kind of 'compensatory growth' when their total mass is reduced; (2) the quantity of bronchial mesenchyme controls the epithelial growth (expressed as total amount of branching), so that when more mesenchyme is present an increased rate of epithelial growth follows.

A more precise interpretation of these results depends on a better understanding of the mechanisms involved in the stimulation. The increase of the measured epithelial surface may be due to at least three different and not incompatible phenomena: (1) the experimental treatments may increase the rate of cell proliferation; (2) they might act on the rate of epithelial change from pseudostratified columnar to simple squamous epithelium at the end of terminal buds, so that the same number of cells may cover a larger epithelial surface; (3) the bronchial cavity size may also increase, simulating effective growth, due for instance to an abnormal accumulation of fluid.

This work is an attempt to correlate the over-all epithelial growth to the mitotic rate of epithelial cells, as measured after colchicine treatment, and to estimate the possible cavity modifications, in order to elucidate some of the

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mechanisms of the apparent growth-stimulating effect noticed in previous experiments. The results obtained show that the rate of growth of the epithelial tree is highly correlated to the mitotic rate under any of the previously quoted experimental conditions, and the characteristics of their correlation were also studied.

MATERIALS AND METHODS

Lung rudiments from 11-day mouse embryos (♀ C57BL × ♂ BALB/c) were placed in hanging-drop culture at the surface of a plasma clot made up with equal amounts of chicken plasma and 9-day chick embryo extract. Three different types of explants were used (Plate 1, figs. 1–6):

Exp. 1. Intact whole lung rudiments.
Exp. 2. Isolated right lung.
Exp. 3. Right primary bronchus in association with whole mesenchyme.

Details on the technical procedures and on the special care taken to avoid superimposition of the bronchial buds and regeneration of the removed part of the epithelial tree (in Exp. 3) were reported earlier (Alescio & Colombo Piperno, 1967). Table 1 gives the experimental plan and numbers of rudiments used.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>+ Colchicine</th>
<th>− Colchicine</th>
<th>Degenerated</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
<td>6</td>
<td>9</td>
<td>45</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>5</td>
<td>13</td>
<td>42</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>5</td>
<td>6</td>
<td>41</td>
</tr>
<tr>
<td>Total</td>
<td>84</td>
<td>16</td>
<td>28</td>
<td>128</td>
</tr>
</tbody>
</table>

Evaluation of epithelial growth in living rudiments. Living cultures were drawn by means of camera lucida after 0, 5, 21, 29 and 45 h of development at 37 °C. The epithelial surface area was measured in arbitrary planimetric units on the drawings. The ratio \(A_t/A_0\) of measurements obtained at 5, 21, 29 and 45 h (\(A_t\)) to the area at zero time (\(A_0\)) was taken as an index of growth (growth factor). This procedure gives homogeneous and readily comparable growth data, independent of the absolute size values of the cultured rudiments at zero time, and of the reduction in the epithelial tree size in Exps. 2 and 3. It should be noted that \(A_t/A_0 = 1\) means absence of growth while, for instance, \(A_t/A_0 = 2\) means that the rudiment at time \(t\) has grown to twice its initial size.

Estimate of mitotic rate. After 45 h the rudiments were subcultured on a new plasma clot containing colchicine (British Drug House, Poole, England) at the final concentration of \(10^{-7}\)M. A small number of rudiments was set aside for the determination of mitotic index in the absence of colchicine, in order to check the effectiveness of the colchicine mitotic block. These were similarly subcultured on a plasma clot in which a corresponding amount of Tyrode solution replaced colchicine.
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The optimal colchicine concentration and length of treatment were determined by trial and error, after testing concentrations ranging from $10^{-5}$ to $10^{-8}$M and exposure times from 4 to 12 h.

After 6 h incubation in the presence of colchicine all rudiments were fixed in Carnoy. Five $\mu$ paraffin sections were cut serially and stained with Regaud's iron haematoxylin.

The following procedure was used to determine the mitotic activity: mitotic epithelial cells were counted in every seventh section of each rudiment; since the straight count of non-mitotic epithelial nuclei proved to be very time-consuming and unreliable, the total number of epithelial cells was estimated by measuring, in arbitrary planimetric units, the epithelial surface present in the same section. Hence, mitotic activity was expressed as number of mitotic cells per surface unit of epithelial tissue. Since a 6 h colchicine treatment was used, the 6 h mitotic rate was estimated.

**Measure of the cavity effect in the determination of over-all epithelial size.** In order to elucidate whether and to what extent bronchial cavity variations participate in the determination of the total size of the epithelial tree, the fraction of the total epithelial surface due to the cavity was measured on histological sections of each rudiment.

**Table 2. Mean values of growth factors and standard errors**

<table>
<thead>
<tr>
<th>Hours</th>
<th>Exp. 1</th>
<th>Exp. 2</th>
<th>Exp. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>21</td>
<td>29</td>
</tr>
<tr>
<td>1</td>
<td>$0.9707 \pm 0.0163$</td>
<td>$1.3769 \pm 0.0294$</td>
<td>$1.6079 \pm 0.0409$</td>
</tr>
<tr>
<td>2</td>
<td>$0.9888 \pm 0.0229$</td>
<td>$1.4846 \pm 0.0386$</td>
<td>$1.7105 \pm 0.0582$</td>
</tr>
<tr>
<td>3</td>
<td>$1.0735 \pm 0.0180$</td>
<td>$1.6624 \pm 0.0232$</td>
<td>$1.8919 \pm 0.0347$</td>
</tr>
</tbody>
</table>

**RESULTS**

The developmental patterns of lung rudiments under conditions created in Exps. 1–3 are shown in Plate 1. Table 2 gives the average values and standard errors of growth factors observed after 5, 21, 29 and 45 h of growth. These values are plotted as a function of time in Text-fig. 1, where the growth curves of the living rudiments from zero to 45 h development in Exps. 1–3 are shown (dashed lines). They show that the experimental treatments 2 and 3 are effective in enhancing epithelial growth well above that of Exp. 1. In fact, an analysis of variance of the average 45 h growth factors (last point of the curves in Text-fig. 1) shows that the differences in the three experiments are highly significant (Table 3). Moreover, in order to analyse closely the differences in growth rate, the linear regression lines were calculated from the average values of growth factors in each of the three experiments (Text-fig. 1, continuous lines). The estimated values of the regression coefficients of growth on time and 5%
confidence intervals (Text-fig. 1) indicate that the whole lung rudiments (Exp. 1) increase their epithelial surface at an hourly rate between 0.0266 and 0.0308. Growth rate is between 0.0340 and 0.0372 in the case of the isolated right lung (Exp. 2), and it reaches 0.0386–0.0418 in the case of the right lung in association with the whole mesenchyme (Exp. 3). A comparison of the regression coefficients by means of Student’s t test shows that the differences are highly significant (P < 0.01) in each comparison.

We may therefore conclude that: (1) substantial differences in growth rate, due to the effect of the experimental treatments, are present; (2) at the beginning of the colchicine treatment the rudiments in the experimental groups are growing at different rates.
Lung rudiments explanted at the 11th day of gestation and cultured for 45 h. Photographs taken in the living state, ×25. Fig. 1: Exp. 1, at starting time. Fig. 2: Exp. 1, at 45 h. Fig. 3: Exp. 2, at starting time. Fig. 4: Exp. 2, at 45 h. Fig. 5: Exp. 3, at starting time. Fig. 6: Exp. 3, at 45 h.
These results are in good agreement with those previously reported (Colombo Piperno, 1966; Alescio & Colombo Piperno, 1967). We also conclude that the differences in growth rates between the experimental groups persist during the next 6 h, since in the previous experiments a linear rate of growth was proved to be maintained for at least 52 h.

The results of the study of mitotic activity are as follows. It is necessary to note first that colchicine treatment for 6 h, at the concentration used in this work, had no harmful effects on the survival of the cultured rudiments; it was, on the other hand, very effective in increasing the number of mitotic figures. Plate 2 shows an example of the histological appearance of colchicine-treated lung rudiments.

### Table 3. Mean value of 45 h growth factors in Exps. 1–3. Analysis of variance

<table>
<thead>
<tr>
<th>Sources of variation</th>
<th>Deviance</th>
<th>D.F.</th>
<th>Variance</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between experiments</td>
<td>128.9678</td>
<td>2</td>
<td>64.4839</td>
<td>363.4943</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Within experiments</td>
<td>17.2091</td>
<td>97</td>
<td>0.1774</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 4. Mean value and 5% confidence interval of mitotic index and mitotic rate

<table>
<thead>
<tr>
<th></th>
<th>Exp. 1</th>
<th>Exp. 2</th>
<th>Exp. 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitotic index</td>
<td>0.0849 ± 0.0141</td>
<td>0.0672 ± 0.0080</td>
<td>0.0928 ± 0.0070</td>
</tr>
<tr>
<td>Mitotic rate</td>
<td>0.1619 ± 0.0245</td>
<td>0.1787 ± 0.0489</td>
<td>0.1785 ± 0.0263</td>
</tr>
</tbody>
</table>

Table 4 gives the average mitotic index with 5% confidence intervals, measured as number of mitotic epithelial cells per surface unit of epithelial tissue, in control cultures in the absence of colchicine, and the mitotic rate after colchicine treatment for 6 h. It appears that no significant differences of mitotic index are present in the three experiments in the absence of colchicine, while a slight increase of mitotic rate is seen from Exp. 1 to Exps. 2 and 3. The latter two do not differ clearly from each other. These small differences are not statistically significant.

In spite of this fact it is of interest to turn from a comparison of average data of each experimental group, to an individual examination of behaviour of mitotic rates, as a function of over-all growth rate of the epithelial tree, within each experimental group. Therefore, the individual regression coefficients of epithelial growth with time were calculated. The mitotic counts after the colchi-
cine treatment give an estimate of mitotic rate in each embryonic lung. Hence, two different and independent assessments of growth become available for each rudiment: (1) an estimate of the regression coefficient which describes its growth measured as increase of the epithelial surface in the living state; (2) an estimate of the mitotic rate over 6 h, measured on the histological sections as the number of mitotic epithelial cells per surface unit of epithelial tissue. It is thus possible to study whether one variable is dependent on the other.

The values of the regression coefficients are plotted as a function of the corre-

Text-fig. 2. The linear regression coefficients of global growth on time are plotted as a function of the corresponding values of mitotic rate. a, Exp. 1; b, Exp. 2; c, Exp. 3; d, data from Exps. 1–3 pooled together. The average values of cavity fraction (with standard errors $s/\sqrt{n}$) in Exps. 1–3 are also shown in d.
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Corresponding values of mitotic rates in Text-fig. 2. They show the trend of the higher mitotic rates to be associated with the higher values of regression coefficients. The degree of correlation may be calculated. The estimated values of correlation coefficients \( r \), given in Table 5, demonstrate a highly significant positive correlation of mitotic rates to regression coefficients of the living cultures. In other words, the higher rates of growth of the living rudiments are definitely associated with higher rates of mitotic activity.

Table 5. Estimates of correlation coefficients \( r \) of mitotic rate to growth rate (regression coefficient) in living rudiments

<table>
<thead>
<tr>
<th>Experiments</th>
<th>D.F.</th>
<th>( r )</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28</td>
<td>0.5843</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>0.8083</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>0.7083</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Pool</td>
<td>82</td>
<td>0.6506</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

In the case of Exp. 1 (Text-fig. 2a) the scattered points may suggest a simple linear dependence of the over-all epithelial growth rate on mitotic activity. The linear regression equation was calculated, and fitting of the linear regression line to the experimental points was tested by Fisher's \( F \) test. The result \( F = 14.5149, P < 0.01 \) shows that the hypothesis of a linear relationship may be accepted. The existence of a linear component in the relationship of the regression coefficients to the mitotic rates was also similarly proved in the case of Exp. 2 \( F = 41.4849, P < 0.01 \) and Exp. 3 \( F = 28.1975, P < 0.01 \). The linear regression lines are drawn in Text-figs. 2a–c).

A similar result is also observed in Text-fig. 2d, where all the data from Exps. 1–3 are pooled together to demonstrate variations of regression coefficients as a function of a larger range of mitotic rates. From the curve in Text-fig. 2d a linear relation of over-all epithelial growth to mitotic rate is also suggested, and it was found to be statistically significant \( F = 60.1860, P < 0.01 \).

The apparently paradoxical conclusion that a low rate of epithelial growth may be expected even in the absence of any epithelial cell proliferation may be deduced from an examination of the curves in Text-fig. 2. This fact is consistent with the hypothesis that other factors, independent of the prominent mitotic activity, participate in the mechanisms of over-all epithelial growth of living rudiments.

Modifications of the tracheo-bronchial cavity may now be considered. Text-fig. 2d shows the mean value of the fraction of epithelial surface due to the tracheo-bronchial cavity in Exps. 1–3. The relative cavity size appears about equal in all cases, which would indicate that the size of the cavity does not affect the estimation of growth. However, the correlation of the cavity fraction to the over-all growth was calculated, using individual measurements of each
cultured rudiment. The estimated values of $r$, given in Table 6, show that no correlation exists in Exps. 1 and 2, as expected. A significant degree of correlation exists in Exp. 3; it is, however, a negative correlation, meaning an inverse dependence of over-all growth on cavity size. In other words, when the cavity fraction increases, the over-all growth rate of the epithelial tree becomes smaller.

We may therefore conclude that an increase in cavity size may affect lung development by depressing its budding rate; in no case does an increase in cavity size take the appearance of growth.

**Table 6. Estimates of correlation coefficients ($r$) of relative cavity size to growth rate in living rudiments**

<table>
<thead>
<tr>
<th>Experiments</th>
<th>D.F.</th>
<th>$r$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28</td>
<td>-0.2046</td>
<td>n.s.</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>-0.2544</td>
<td>n.s.</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>-0.4987</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

**DISCUSSION**

It is noteworthy to remark first on the high degree of reproducibility in the effects of the Exps. 2 and 3, which fully confirm the results previously obtained (Colombo Piperno, 1966; Alescio & Colombo Piperno, 1967). It seems quite clear that, under the reported conditions of growth in vitro, both mass reduction and an increased proportion of mesenchyme are effective in enhancing the over-all epithelial growth rate significantly above the level of control rudiments. We have now shown that this effect is mainly due to a stimulation of epithelial cell mitotic activity.

No attempt was made in this work to analyse closely the kinetics of epithelial cell proliferation in the bronchial epithelium of developing lungs. The data presented here are intended only to give a comparative assessment of mitotic rates in different experimental conditions as a function of over-all growth rate of the living rudiments.

In the control group of lung rudiments (Exp. 1) a highly significant degree of correlation exists between over-all growth rate and mitotic rate. This means that mitotic activity is very important in the control of the budding rate of living rudiments. When we perform experiments in which part of the rudiment is isolated (Exp. 2), or part of the epithelial tree is brought under the influence of the total amount of mesenchyme (Exp. 3), the epithelial growth rate is significantly and constantly enhanced, and at the same time a higher rate of epithelial cell division is observed. Since the degree of correlation persists, and even improves, in Exps. 2 and 3, it is clear that mitotic activity is correspondingly enhanced. The experimental treatments are therefore capable of stimulating, ultimately, the mitotic activity.
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That differences in the average mitotic rate of the three experimental groups are not statistically significant does not affect this statement, since a high degree of correlation persists. The reason for non-significant differences in mean mitotic rate is most likely to be that the colchicine treatment must be limited to a short length of time (6 h), which represents but a small fraction of the growth time required to bring about differences ample enough for statistical significance. Our preliminary experiments showed that the colchicine treatment under our experimental conditions could not be safely continued for longer than 6 h; also the possibility of a toxic effect affecting the entering of cells into mitosis (Stevens Hooper, 1961), as well as the reversal of the mitotic block (Kleinfeld & Sisken, 1966) must be considered.

It may be noted that the use of the epithelial surface in embryonic lungs developing in vitro may be more a sensitive tool for growth assessment than determination of mitotic rate by colchicine treatment.

We may conclude that under the reported conditions of growth in vitro, the over-all growth of the epithelial surface area is closely related to the mitotic activity of the epithelial cells. At least part of the growth-enhancing effect due to a mass reduction and to an increased amount of mesenchyme in the cultured rudiments seems now to be due to an increased rate of cell division. Cavity size modifications, even if present, do not interfere with growth assessment of the epithelial tree; on the contrary, an increase of cavity fraction, being inversely dependent on the global size of the epithelial tree, would eventually cause an over-all reduction of the growth rate.

The third phenomenon envisaged above as a tentative explanation of the growth-stimulating effect, namely an accelerated rate of the normal flattening of the bronchial epithelium, may be present as a co-factor of growth acceleration. We have no direct experimental data regarding this particular point, but our results suggest that other factors besides cell division determine growth rate in living rudiments, and they appear to be also enhanced by our experimental treatments. We may therefore expect that the lung mesenchyme not only controls the epithelial mitotic activity, but may also promote other activities stimulating growth and differentiation.

An effect of the amount of mesenchyme on epithelial growth has already been noted but not quantitatively estimated (Sobel, 1958; Dameron, 1962; Wessells, 1963; Rutter, Wessells & Grobstein, 1964); also a dependence of epithelial growth on the total size of the rudiment was recently noticed and discussed by Wessells & Cohen (1967) in pancreatic rudiments grown in vitro.

While it is more difficult to suggest an explanation of how mass reduction can affect the epithelial growth rate, a tentative explanation of the mechanisms involved in the mesenchymal stimulation of epithelial growth may involve lung mesenchyme producing or containing protein substances related to the 'epithelial growth factor' (E.G.F.) which has been shown to regulate the proliferation rate of several epithelia (Cohen, 1964, 1965; Jones, 1966).
SUMMARY

1. The rate of cell division was studied as a function of over-all growth of the epithelial tree of the 11-day mouse embryonic lung developing in vitro, under conditions of: (1) normal growth in vitro of intact rudiments; (2) mass reduction; (3) increase of the relative amount of mesenchyme.

2. The over-all rate of epithelial growth under experimental situations (2) and (3) is significantly higher than that of intact rudiments.

3. The epithelial cell proliferation rate is highly correlated with the over-all size of epithelial growth in all cases.

4. It was concluded that the over-all growth and budding activity of the epithelial tree depends on cell division. Reduction in mass and an increased amount of mesenchyme increase the rate of epithelial growth by stimulating the mitotic activity of the epithelial cells.

RIASSUNTO

Relazione dell'accrescimento epiteliale con il tasso mitotico nel polmone embrionale di topo coltivato in vitro.

1. Si è studiato il tasso di divisione cellulare in funzione dell'accrescimento globale nell'albero epiteliale del polmone embrionale di topo di 11 giorni di gestazione, coltivato in vitro nelle seguenti condizioni: (1) accrescimento normale in vitro di abbozzi coltivati interi; (2) riduzione della massa totale dell'abbozzo; (3) aumento relativo della quantità di mesenchima.

2. Il tasso di accrescimento globale nelle condizioni sperimentali di cui ai punti (2) e (3) è significativamente maggiore che negli abbozzi intatti.

3. Il tasso di proliferazione cellulare è strettamente correlato con il tasso di accrescimento epiteliale complessivo in tutte le tre condizioni sperimentali.

4. Si conclude che l'accrescimento globale e la gemmazione degli abbozzi polmonari dipendono dalla divisione cellulare. La riduzione della massa dell'organo e l'aumento relativo del mesenchima aumentano il tasso di accrescimento globale stimolando l'attività proliferativa delle cellule epiteliali.

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