The influence of mesenchyme on the epithelial glycogen and budding activity in mouse embryonic lung developing \textit{in vitro}

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SUMMARY

Experiments have been performed to show the association of epithelial glycogen with budding activity, and their dependence on the nature of the associated mesenchyme. The bronchial mesenchyme associated with the tracheal epithelium induces the appearance of a tracheal bud where glycogen remains present after its removal from the rest of the trachea. The bronchial mesenchyme associated with the tracheal epithelium after the normal disappearance of glycogen, still induces the formation of an epithelial bud where glycogen is clearly demonstrable. The primary left bronchus completely ceases its budding activity when brought in association with metanephrogenic mesenchyme; glycogen is totally absent from the resting epithelium. The reassociation of the morphogenetically quiescent primary left bronchus with bronchial mesenchyme brings about resumption of budding activity and reappearance of the epithelial glycogen, showing normal bronchial distribution.

INTRODUCTION

The aim of this work is to present some evidence that influences from the mesenchymal environment may act on one of the specific metabolic patterns of the pulmonary epithelium. It will be shown that the ability of the epithelial cells to accumulate glycogen is dependent on the nature of the mesenchymal environment, as it can be modified by displacements of parts of the pulmonary mesenchyme, and by partial exchange of heterologous (metanephrogenic) mesenchyme for homologous (bronchial) mesenchyme. The effect of such displacements and exchanges operated in the mesenchymal mantle is strictly localized to the epithelial area immediately underneath the transposed mesenchymal mass. Evidence that the presence of glycogen is associated with budding activity will also follow.

As far as the pulmonary epithelium is concerned, it has been shown that glycogen is predominantly localized in the branching regions, and that it leaves the older portions of the epithelial tree as it appears distally; consequently the

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terminal buds always present the highest glycogen concentration (Sorokin, Padykula & Herman, 1959; Sorokin, 1961). Taking advantage of this topographical association of glycogen with the budding regions, we have asked: (1) whether glycogen will be present in the tracheal epithelium when induced to bud by grafted bronchial mesenchyme (Alescio & Cassini, 1962a; Borghese, Alescio & Cassini, 1963); (2) whether the presence of glycogen in the terminal buds is specifically dependent on the nature of the surrounding mesenchyme.

Fig. 1. Experimental programme. BM (dotted areas) = bronchial mesenchyme; MM (dashed areas) = metanephrogenic mesenchyme.

MATERIALS AND METHODS

First-generation mouse hybrids from ♀ C57BL × ♂ BALB/c were used. Lung rudiments were explanted at the 11th day of gestation and cultured in hanging drop on a plasma clot composed by equal volumes of chicken plasma and 9-day chick embryo extract.
Experiments were performed as follows:

1. Grafting of bronchial mesenchyme (BM) on the tracheal epithelium of 11-day lung rudiments immediately after explantation.

2. Grafting of 11-day BM on the tracheal epithelium of 11-day rudiments precultured for 1 day (total age at grafting time = 12 days).

3. Grafting of 11-day metanephrogenic mesenchyme (MM) on the left primary bronchus of 11-day rudiments, immediately after explantation.

4. Grafting of MM on the left primary bronchus of 11-day rudiments as in experiment 3; after 2 days in culture the MM was removed and freshly dissected 11-day BM was substituted.

In all experiments the tissues to be grafted or removed were detached by simple dissection, avoiding the use of trypsin and of any other dissociating chemical agent; the BM for grafting was always taken from 11-day rudiments, to ensure maximum inductive effect.

The experimental programme is reproduced in the schemes in Fig. 1, where for simplicity the epithelial budding developed during the cultivation period is not reproduced.

Table 1 shows the total number of cultures in each experiment.

All rudiments were fixed in Carnoy, serially sectioned at 5 μ and stained by the periodic acid-Schiff (PAS) sequence after McManus (Pearse, 1968); the specificity of the glycogen staining was controlled by diastase digestion after Lillie (1965).

The normal distribution of glycogen in the lung rudiments during in vivo development was studied in controls at the 11th and 12th day of gestation, using the above-mentioned procedure.

RESULTS

The glycogen distribution in the 11-day lung rudiment from mouse embryos is shown in Fig. 2. The glycogen is present and more or less evenly distributed in the whole epithelial tree, but quickly disappears from the epithelium of the trachea and primary bronchi during the next 24 h in culture, after which it remains conspicuously present only in the terminal buds, as shown in Fig. 3. This behaviour is not due to culture conditions, but rather represents the normal temporal pattern of glycogen distribution, since it corresponds closely to what is seen in lung rudiments from 12-day embryos, that is at the same total age. These results are in agreement with the data already obtained with the embryonic lung of several mammalian species (reviewed by Sorokin, 1965).

Experiment 1. Effect of bronchial mesenchyme grafted on tracheal epithelium.

The effect of this type of experiment is shown in Fig. 4: an abnormal bud frequently develops from the tracheal epithelium (Table 1) under the influence of the grafted bronchial mesenchyme.

The PAS reaction shows that glycogen is conspicuously present in the epithelium of the tracheal bud (Fig. 5), while completely absent, as far as may be
Fig. 2. Eleven-day lung rudiment. Presence of glycogen in the tracheo-bronchial epithelium. PAS-haematoxylin. \( \times \) 125.

Fig. 3. Eleven-day lung rudiment cultured for 1 day. Presence of glycogen only in the terminal buds. PAS-haematoxylin. \( \times \) 125.

Fig. 4. Eleven-day lung rudiment cultured for 2 days. A fragment of BM grafted on trachea has induced the appearance of a tracheal bud (tb). Photograph in the living state. \( \times \) 24.

Fig. 5. Histological section of the tracheal bud reproduced in Fig. 4. Presence of glycogen in the budding epithelium. PAS-haematoxylin. \( \times \) 125.

Fig. 6. Eleven-day lung rudiment cultured for 3 days. A fragment of BM grafted on trachea after 1 day in culture has induced the appearance of a tracheal bud (tb). Photograph in the living state. \( \times \) 24.

Fig. 7. Histological section of the tracheal bud reproduced in Fig. 6. Presence of glycogen in the budding epithelium. PAS-haematoxylin. \( \times \) 200.
assessed histochemically, in the remaining portions of the tracheal epithelium. This result is constant and qualitatively fully reproducible, in the sense that when a tracheal bud is produced, glycogen is always clearly present in it (Table 1), although probably quantitatively different, as the amount of PAS-positive diastase-sensitive material appeared variable from case to case.

Table 1. Number of rudiments and results

<table>
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<th>Exp.</th>
<th>Budding Glycogen</th>
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<td>3</td>
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<td>12</td>
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<td>4</td>
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<td>1</td>
<td>1</td>
<td>19</td>
<td>10</td>
<td>57</td>
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It is therefore concluded that, when glycogen is still present in the tracheal epithelium at the time of grafting, the tracheal epithelium either maintains its ability to synthesize glycogen, or does not discharge glycogen, when induced to bud under the influence of bronchial mesenchyme.

Experiment 2. Grafting of BM on the tracheal epithelium of 11-day rudiments precultured for 1 day. In order to distinguish between the two possible interpretations of the preceding experiment (continued synthesis and/or no discharge of glycogen) BM was grafted on the tracheal epithelium after one day in culture, that is when glycogen has been completely cleared from the epithelial cells. The results presented in Table 1 show that the tracheal epithelium after one day in culture is still competent to produce abnormal budding under the influence of the bronchial mesenchyme, as the formation of the tracheal bud was obtained in twelve rudiments out of sixteen grafted. An example is shown in Fig. 6. Moreover, the epithelial cells of the newly formed tracheal bud again show clearly the presence of glycogen (Fig. 7), with the exception of one rudiment (see Table 1) where the tracheal bud showed only traces of PAS-positive material.

The bronchial mesenchyme is therefore able to induce not only budding but also the reappearance of glycogen in the tracheal epithelium well after its normal disappearance.

Incidentally we point out that the ability of the tracheal epithelium to bud under the influence of the bronchial mesenchyme is apparently lost after one more day in culture. In fact in a collateral experiment the attempt to obtain tracheal budding after 2 days in culture was almost completely unsuccessful as tracheal budding was obtained in only three rudiments out of eighteen grafted.

Experiment 3. Grafting of metanephrogenic mesenchyme on the left primary bronchus in 11-day rudiments. The foregoing results show that the presence of glycogen in the tracheal epithelium is strictly associated with budding induced
by grafted bronchial mesenchyme. It is therefore of interest to determine in the
normally budding (bronchial) regions of the rudiment whether the presence of
glycogen is also similarly associated with the actual appearance of secondary
bronchi. Fig. 8 shows that the 11-day main left bronchus when deprived of its
own mesenchyme, immediately and completely ceases its budding activity, as no

Fig. 8. Eleven-day lung rudiment at explantation. MM is grafted on the left primary
bronchus. Photograph in the living state. ×24.

Fig. 9. The same rudiment as in Fig. 8 after 2 days in culture. Absence of budding
from the left primary bronchus in the presence of MM. Photograph in the living
state. ×24.

Fig. 10. Histological section of the left primary bronchus after 2 days of culture in
association with MM, at a stage comparable to Fig. 9. No glycogen is seen in the
bronchial epithelium. PAS-haematoxylin. ×200.

Fig. 11. The same rudiment as in Fig. 9. MM has been removed, and BM re-
associated with the left primary bronchus. Photograph in the living state. ×24.

Fig. 12. The same rudiment as in Fig. 11 after 2 days in culture in the presence of
BM. Resumption of budding activity in the left primary bronchus. Photograph in
the living state. ×24.

Fig. 13. Histological section of the left primary bronchus of the rudiment in Fig. 12.
Reappearance of glycogen in the terminal buds. PAS-haematoxylin. ×80.
progress of the bronchial arborization is seen for at least 2 days in culture (Fig. 9), and this result appears very constant (Table 1). The PAS reaction (Fig. 10) indicates that during the phase of morphogenetic rest following the heterogeneous association with \( MM \), no PAS-positive material can be traced in the epithelial cells, with the possible exception of one rudiment where a small amount of glycogen was still recognizable (Table 1).

This result shows that the substitution of \( MM \) for \( BM \) entails total and immediate cessation of budding, associated with a complete disappearance of glycogen.

Experiment 4. Reassociation of 11-day \( BM \) with the left bronchial epithelium after removal of \( MM \). The morphogenetic quiescence brought about by the association of the bronchial epithelium with \( MM \) represents only a temporary effect exerted by the heterologous mesenchyme, and not definitive loss of the morphogenetic potentialities. Indeed, when \( MM \) is removed and 11-day \( BM \) is reassociated with the left bronchus (Fig. 11) budding activity is soon resumed in all cases (Table 1), and 2 days after the homologous reassociation a considerable degree of bronchial branching has been produced (Fig. 12). Again, glycogen has reappeared in the epithelial tree, where it shows the characteristic bronchial distribution with maximal concentration in the terminal buds (Fig. 13).

Altogether these results show that: (1) the epithelial branching in mouse embryonic lung in culture conditions is always associated with the presence of glycogen in the evaginating epithelial cells; (2) influences from the bronchial mesenchyme, which are required to produce the budding movement, also induce glycogen accumulation.

We have not yet direct proof of a causal relationship between the presence of glycogen and the bronchial branching: however, we have data showing that in the association of bronchial mesenchyme with the tracheal epithelium (Exp. 2) reappearance of glycogen may be observed in the tracheal epithelium shortly after grafting, when actual evaginating movement is not yet recognizable. This may indicate, but does not prove, that if glycogen comes first and then the epithelial movement follows, the presence of this polysaccharide may be required for budding.

**DISCUSSION**

Our data suggest the existence of a significant association between the presence of glycogen and bronchial arborization. The data in Table 1 indicate very clearly that budding activity and glycogen are both present, or both absent, under our experimental conditions, with impressive constancy. Technical reasons may well explain the two reported exceptions: for instance, loss during fixation and retarded degradation of glycogen may be responsible. However, quantitative measurements will be necessary to give more conclusive significance to the reported glycogen variations.

While these data seem to imply an organogenetic function of glycogen in
lung development as earlier suggested by Sorokin (1965), they do not necessarily
disprove the view that the embryonic lung may function as a 'preportal liver'
in storing carbohydrate during prenatal life (Fauré-Fremiet & Dragoiu, 1923;
Szendi, 1936; Parhon & Milcou, 1938; Graumann, 1964).

While the problem of the morphogenetic significance of glycogen remains
open, the relevant point in our opinion is that we may now consider the activity
of mesenchyme in induction of epithelial budding as possibly related also to the
appearance of one specific product of the epithelial cells. One of the effects of the
bronchial mesenchyme may therefore consist in the ability to induce synthesis
and/or activation of the enzymic systems participating in glycogen metabolism.
Le Douarin (1968) has shown a similar dependence of glycogen synthesis in the
hepatic endoderm from chick embryos on the nature of the associated mesenchyme.

These data further emphasize the idea of a multiplicity of mesenchymal
factors operating in epithelio-mesenchymal interactions (Grobstein, 1967).
Indeed, during the earlier phases of lung development, characterized by epi-
thelial branching and by a wavelike spread of differentiative changes down the
bronchial tree from the tracheal region to the terminal buds (Sorokin, 1965), we
are confronted with at least three different types of mesenchymal effect experi-
mentally distinguishable:

(1) Control of growth rate, through the regulation of the rate of epithelial
cell proliferation (Colombo Piperno, 1966; Alescio & Colombo Piperno,
1967; Alescio & di Michele, 1968; Alescio, 1968). This is apparently operated by
mesenchymal factors on the whole of the epithelial tree, suggesting that all
epithelial cells are under mitotic control, irrespective of their position in the
epithelial tree, since there is no preferential localization of mitotic figures in the
terminal buds after colchicine treatment (Alescio & di Michele, 1968).

(2) Precisely localized control of the morphogenetic pattern through the
determination of the timing and sites of appearance of the secondary buds, as
shown by the present and, more extensively, by previous results (Alescio &
Cassini, 1962a, b). In this context more recent work by Taderera (1967) and by
Wessells & Cohen (1968) on embryonic lungs and other organs, has emphasized
the importance of collagenase-sensitive materials polymerizing at the epithelio-
mesenchymal interfaces in modelling the shape and geometry of the epithelial
part of the rudiments: the main mechanism would be here of an extracellular
type.

(3) Regulative effect on specific metabolic patterns of the epithelial cells, as
shown in the current paper. If the clearance of glycogen from the epithelium may
be considered a biochemical marker of stabilization of the bronchial structure,
it is clear that the information for such a metabolic switch comes to the epi-
thelium from the surrounding mesenchyme. Evidently the clearance of glycogen
may be achieved through reduced synthesis and/or increased degradation. Work
is now in progress to clarify further the morphogenetic significance of glycogen,
and the nature of its mesenchymal control.
RIASSUNTO

L'influenza del mesenchima sul glicogeno epiteliale e sulla gemmazione nel polmone embrionale di topo coltivato in vitro

Si sono eseguite esperienze allo scopo di mostrare l'associazione del glicogeno epiteliale con l'attività di gemmazione, e la loro comune dipendenza dalla natura del mesenchima associato.

Il mesenchima bronchiale associato all'epitelio della trachea induce la comparsa di una gemma tracheale in cui il glicogeno rimane presente dopo la sua scomparsa da tutto il resto della trachea.

Il mesenchima bronchiale associato all'epitelio della trachea dopo la normale scomparsa del glicogeno induce ancora la formazione di una gemma epiteliale in cui il glicogeno è chiaramente dimostrabile.

Il bronco epiteliale sinistro, associato a mesenchima metanefrogeno, cessa completamente la sua attività di gemmazione; il glicogeno è totalmente assente dall'epitelio durante tale fase di riposo.

La riassociazione del bronco primario sinistro morfogeneticamente quiescente con mesenchima bronchiale provoca la ripresa della gemmazione e la ricomparsa del glicogeno epiteliale, che segue la normale distribuzione bronchiale.

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REFERENCES


(Manuscript received 28 May 1970)