Transfilter induction of kidney tubules as a function of the extent and duration of intercellular contacts

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SUMMARY
The kinetics of kidney tubule induction were examined in transfilter experiments by varying the time of transfilter apposition, the porosity and pore size of the filters, and the culture conditions. Transfilter contact between the interacting cells is established within an hour when cytoplasmic processes emerge through the interposed filter; then a further 16–24 h are needed for completion of induction. This lag is a function of thickness and pore size of the filter, and is not reduced by precultivation of the inductor on the filter. Material that accumulates on the far side of the filter during such cultivation displays no morphogenetic activity.

The intensity of the mesenchymal response was roughly quantified and shown to be a function of pore size, pore density and duration of transfilter contact.

INTRODUCTION
Various mechanisms have been postulated for the transmission of inductive signals between embryonic cells (Grobstein, 1956; Saxén, 1972). Recently we found it convenient to distinguish between two major types of transmission, long-range and short-range. The former refers to transmission over distances of the order of 50000 nm, the latter to an interaction through close cell contacts up to about 5 nm (Saxén, 1977). The interaction leading to kidney tubule formation, first demonstrated by Grobstein (1953), apparently belongs to the latter category. In the kidney rudiment the basal lamina separating the interacting epithelial and mesenchymal cells has discontinuities, and through these the cells come into close contact, with only a few nm between their plasma membranes (Lehtonen, 1975). The results of experiments with interposed filter membranes of varying pore size suggest that the inductive stimuli are transferred only after close contact has been established between the inductor and the target cells (Wartiovaara, Nordling, Lehtonen & Saxén, 1974, Lehtonen, Wartiovaara, Nordling & Saxén 1975, Saxén et al. 1976b). The mode of interactions at these sites has remained unknown.

The experiments reported here were designed to shed further light on the

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significance of the close intercellular contacts believed to be the sites of inductive interactions. A rough quantitative method was devised to estimate the intensity of the response in the target tissue, and this was used for evaluating the temporal requirements of contact-mediated induction. The same method was used to investigate the significance of the number of contact sites and the area of contact. The method also allowed us to test whether inductive capacity persists in a 'membrane preparation' devoid of functional nuclei.

MATERIALS AND METHODS

Tissues

Hybrid mouse embryos BALBc × CBA were removed from the uterus on day 11 (0 being the day of appearance of the vaginal plug). Following Grobstein's (1955) original model-system, spinal cord was used as a potent and convenient inductor. The ureter bud was separated from the undifferentiated metanephric mesenchyme after short treatment with EDTA. Pieces of spinal cord were trimmed to the size of one mesenchyme and all tissues were pre-incubated for 3 h in the culture medium before explants were set up.

Transfilter cultures

The spinal cord fragment was cemented to the lower (matt) surface of the Nuclepore filter with 1% agar, and the mesenchyme was placed on the opposite side. The explant was grown on a metal screen in a Trowell-type culture (Saxén & Saksela, 1971). The culture medium was Eagle's minimum essential medium in Earle's balanced salt solution supplemented with 10% foetal calf serum (Microbiological Associates Inc., Bethesda, Md.). Unless otherwise stated, the explant was fixed after culture for 72 h.

Filters

Nuclepore® filters provided by the General Electric Co. (Pleasanton, Cal.) were used throughout the work. Commercial grade filters were used with nominal pore sizes of 0.05 μm, 0.1 μm, 0.2 μm and 0.6 μm. The corresponding pore densities were $6 \times 10^8$, $3 \times 10^8$, $3 \times 10^8$ and $3 \times 10^7$ pores/cm². In addition, the company provided upon request two special filters with a pore size of 0.6 μm, but with different pore densities, $2 \times 10^9$/cm² and $2.3 \times 10^7$/cm². These special filters will be referred to as 'low' and 'high' porosity filters. Before use the filters were sterilized in 70% alcohol, thoroughly rinsed in PBS, and kept in the culture medium.
Fig. 1. Micrographs of whole-mount preparations of kidney mesenchyme exposed transfilter to spinal cord under various conditions. The four explants demonstrate the quantitatively varying formation of tubules.

**Whole mount preparation for quantitative estimates**

Since no specific metabolic features are known by which the early stages of tubule formation can be identified (Saxén et al. 1968), the only way to quantify the response was to measure the final result of induction. Each explant comprised one 11-day mesenchyme dissected out before the first branching of the ureter bud and combined transfilter with a piece of trimmed spinal cord. After the standard culture time, the explant was fixed in formal solution, the inductor was removed, and the whole mesenchyme was mounted according to the method of Gossens & Unsworth (1972). The numbers of tubules in these mounts (Fig. 1) was counted.
Fig. 2. Micrograph of a 1 h culture with spinal cord below the Nuclepore filter (pore size 0.2 μm), and mesenchyme on top of it. Abundant cytoplasmic material is seen in the pores. H & E stain.
Transfilter tubule induction

Light and electron microscopy

The cultures were rinsed in PBS and fixed in 2.5% glutaraldehyde in 0.15 M cacodylate buffer, pH 7.6, for 45–60 min at room temperature. For transmission microscopy, the explants were postfixed in 1% OsO4 in 0.1 M phosphate buffer, pH 7.3, for 60 min at 4 °C and embedded in Epon 812. Thick Epon sections for light microscopy were stained with 1% toluidine blue in 1% borax. Thin sections were stained with uranyl acetate and lead citrate, and examined in a Jeol JEM-100B transmission electron microscope. For scanning electron microscopy, the fixed explants were dehydrated in ethanol and critical point dried through amyl acetate and CO2. The samples were covered with a thin layer of carbon and gold in a Balzer Micro-BA3 evaporator and examined in a Jeol JSM-U3 scanning electron microscope.

RESULTS

Establishment of transfilter cell contacts

As previous experiments had shown that transfilter induction is a time-consuming event (Wartiovaara et al. 1974), it seemed relevant to examine the kinetics of the process by which the cells establish contacts through the 10 μm-thick filter.

Filters were fixed after 1, 2, 6, 12 and 30 h in experiments of two types, the sandwich type with tissues on both sides of the filter and those with only the inductor on the filter membrane. In all 15 filters with 0.6 and 0.2 μm pores cytoplasmic material was detectable, and after as little as an hour such material could be seen to have grown right through the filter and in practically every pore (Fig. 2). The result was the same when the filter was set up with the inductor alone. Prolonged culture (12 h) led to accumulation of stainable material on the far side of the filter.

In electron microscopy, membrane-coated cytoplasmic processes were regularly seen in channels down to 0.2 μm, after cultivation for 1 h (Fig. 3B). In the smallest pores examined (average pore diameter 0.1 μm), the finding was less consistent even after a longer culture period. After an hour, processes frequently penetrated right through this filter (Fig. 3A) but many of the pores remained empty. After 30 h, penetration was usually ample in some areas (Fig. 4), but entirely negative results were not rare. The stainable material accumulating on the opposite filter surface proved to consist of the large, bulging tips of the cytoplasmic processes (Figs. 3 and 4).

Passage of inductive signals through various filters

Twenty-eight sandwiches grown on commercial filters with average pore sizes of 0.6 and 0.2 μm invariably yielded positive results when cultured for 72 h. With both pore sizes the mean number of tubules per metanephric mesenchyme was 20.
Fig. 3. Electron micrographs of spinal cord tissue sending filamentous processes into the channels of Nuclepore filters with an average pore diameter of 0.1 µm (A) and 0.2 µm (B). The culture time is 1 h, and bulging processes are seen protruding through the filter.
With the filters with pores of 0.1 μm, contacts increased when we replaced the large piece of spinal cord used earlier with a small, trimmed fragment, and the passage of inductive signals was considerably improved as compared to our original series (Wartiovaara et al. 1974). Of the 46 explants, 26 developed tubules. The response was still weak and the mean number of tubules in these positive explants was 3.1.

Twelve experiments were made with a Nuclepore filter with pores of 0.05 μm. No tubules were seen in these mesenchymes.

*Induction as a function of pore density*

Following the suggestion of Meier & Hay (1975) that induction might be a function of pore density, i.e. of the total contact area of the interacting tissues, we tested the ‘low’ and ‘high’ porosity filters with 0.6 μm pores. The response of the mesenchyme was measured from the whole mounts (Table 1). Both the
Table 1. Induction of kidney tubules in the metanephric mesenchyme through Nuclepore filters of 'low' and 'high' porosity (average pore size 0.6 μm)

<table>
<thead>
<tr>
<th>Porosity (pores/cm²)</th>
<th>Positive cases</th>
<th>Mean number of tubules</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>2 x 10⁶</td>
<td>14/27</td>
<td>52</td>
</tr>
<tr>
<td>2.3 x 10⁷</td>
<td>20/20</td>
<td>100</td>
</tr>
</tbody>
</table>

Fig. 5. Diagram showing the minimum induction time expressed as a percentage of positive explants, with two types of filter. In one set of experiments with a 0.6 μm pore size filter the inductor was precultivated for 24 h. Based on altogether 250 explants.

number of positive explants and the average number of tubules in them increased considerably with an increase in pore density. In further experiments, in which the spinal cord was precultivated on the 'low' porosity filter for 48 h to allow good penetration before application of freshly dissected mesenchyme, precultivation did not improve the passage of the signals. Of ten such explants, four yielded tubules, their mean number being 5.0.

Minimum induction time after precultivation

Previously, the minimum induction time had been estimated by removing the inductor after different time intervals (Wartiovaara et al. 1974, Saxén et al. 1976b). Depending on the type of filter, this minimum time was somewhere...
between 12 and 30 h. Since the rapid penetration of cytoplasmic material through the pores suggested that this long lag period was not due to slowness in establishing contact, we investigated whether the minimum time could be shortened by a 24 h pre-cultivation of the inductor or the target tissue. This would, for example, allow the tissues to recover from the manipulations and regain their surface-associated materials. The results (Figs. 5 and 6) showed that:

The minimum induction time (50% of explants induced) is of the order of 16 h for the 0.6 μm filter and some 8 h longer for the 0.2 μm filter. Because of inconsistent results, no minimum induction time was determined for the 0.1 μm filter.

The response at tissue level is not an all-or-none phenomenon, for both the proportion of positive explants and the mean number of tubules increase with increasing pore size and with prolongation of the transfilter contact.

Precultivation of the inductor tissue does not detectably affect the results.

In 20 similar experiments with mesenchymes precultivated for 24 h, there was likewise no shortening of the minimum induction time.

**Minimum induction time as a function of filter thickness**

Adopting our earlier experimental design with Millipore filters (Nordling, Miettinen, Wartiovaara & Saxén, 1971), we made experiments with 0.6 μm Nuclepore membranes to establish whether the minimum induction time and the intensity of the response are functions of the distance between the inter-
Table 2. Induction of kidney tubules in the metanephric mesenchyme as function of transfilter contact time and filter thickness. (Nuclepore filters with average pore diameter of 0.6 \( \mu \)m)

<table>
<thead>
<tr>
<th>Contact time (h)</th>
<th>Single filter</th>
<th>Double filter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive explants</td>
<td>Tubules per positive explants</td>
</tr>
<tr>
<td>10</td>
<td>0/10</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>1/8</td>
<td>7.1</td>
</tr>
<tr>
<td>16</td>
<td>14/20</td>
<td>15.2</td>
</tr>
<tr>
<td>24</td>
<td>15/15</td>
<td>2/10</td>
</tr>
<tr>
<td>30</td>
<td>10/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>

acting cells. The results (Table 2) indicate an affirmative answer: When an additional filter was placed between the spinal cord and the responding mesenchyme, the minimum induction time was prolonged by some 12 h and the response was weaker even after a contact of 72 h.

Failure to induce with a 'membrane preparation'

The knowledge that in certain interactive situations extracellular materials exert morphogenetic influences (e.g. Lash & Vasan, 1977) and that precultivation of our inductor produced cytoplasmic material on the opposite filter surface led us to test whether material without nuclei would induce tubule formation. Therefore, the spinal cord was carefully removed from the filter after 24 h of precultivation. This was done without touching the other surface, where material derived from the spinal cord could be demonstrated (Fig. 7 A). During subsequent cultivation this material gradually disintegrated but 24 h later definitive remnants of the bulging processes were still detectable (Fig. 7 B). Metanephric mesenchyme was placed on this material immediately after removal of the spinal cord and the explants were subcultured for another 72 h. None of the 15 mesenchymes so exposed showed signs of tubule formation.

DISCUSSION AND CONCLUSIONS

As the basis of our experiments we assumed that induction of tubules in the metanephric mesenchyme is mediated by actual contacts between cells rather than by long-range passage of signal substances. If interpreted from this viewpoint the results lead to the following conclusions:

Membrane filters with pores ranging from 0.6 to 0.2 \( \mu \)m in diameter allow rapid contact between interacting cells. Cellular processes grow through the filters in one hour.
Fig. 7. Upper surface of Nuclepore filter of 0.6 μm pore size. Spinal cord was cultured on the lower filter surface for 24 h, then removed. (A) Immediately after removal of the spinal cord tissue from the opposite side cytoplasmic processes comparable to those in Figs. 3 and 4 were regularly seen. (B) After subculture for 22 h without spinal cord. Remnants of processes derived from the spinal cord are still detectable.
The rate of growth of cytoplasmic material into the filter pores did not detectably differ through pores with diameters from 0.6 to 0.2 µm.

Induction requires a long minimum contact time of the order of 16 h for 0.6 µm filters and 24 h for the 0.2 µm filters.

Precultivation of the inductor on the filter does not shorten the minimum induction time, suggesting that this is not determined by operative artefacts, such as removal of surface compounds.

Induction at tissue level is not an all-or-none phenomenon but quantitatively dependent on the duration of contact, and also on the size and density of the contact sites.

If transmission could occur only through specialized membrane structures such as gap junctions, the time- and area-dependence would result from the small surface area occupied by such junctions (Gilula, 1977) and from their relatively slow formation (Johnson, Hammer, Sheridan & Revel, 1974). With increasing size and number of contact sites and with increasing time of close apposition the chances of developing junctional structures would increase. One could as well speculate that the compounds synthesized by the inductor cells might have different transmission velocities in the 10 µm long processes varying in diameter from 0.1 µm to 0.6 µm, which means a 30-fold difference in cross-sectional area.

The hypothesis that active compounds are transmitted through intimate contacts is further supported by the observation that isolated cytoplasmic and membrane components did not have an inductive effect in our system, as they do in some other interactive models (for review, see: Saxén et al. 1976a). This failure does not necessarily mean that surface associated compounds play no part in the induction of kidney tubules.

The skilful technical assistance of Miss Pirkko Leikas and Mrs Anja Tuomi is acknowledged. This work was supported by the Finnish Culture Foundation and the Sigrid Jusélius Foundation, Finland.

REFERENCES


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(Received 20 February 1978, revised 25 April 1978)