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Effects of Rathke's Pouch Mesenchymal Cells on Growth Hormone and Prolactin Release from Pituitary Clonal Cells (2E6)[1] (40176)

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Evidence that normal embryological development of many organs depends on interactions between epithelial and mesenchymal tissue has been well established (1). Some epithelial components require their own particular mesenchyme, while others, i.e., pancreatic epithelia, develop with mesenchyme from a variety of sources (1, 2). Rutter's group proved that when extracts from mesenchymal tissue were added to the culture medium, normal rat pancreatic epithelia were stimulated in development (2–6). Recently Pantazis *et al.* (7) demonstrated that L clonal fibroblast cells (mouse L-929 cells, subline L2) derived from loose connective tissue secreted a nerve growth factor.

It has been reported that pituitary anlage cells differentiate automatically in the fetus even after encephalectomy (8–13). However, it is very difficult to determine whether or not pituitary anlage cells differentiate automatically since it is impossible to remove mesenchymal cells from the epithelial cells of Rathke's pouch when one separates these tissues. Tixer-Vidal (14) demonstrated that pituitary cells apparently require contact with fibroblasts. These fibroblasts may initially act as a natural feeder layer, but they later limit the survival of the glandular cells because of their high mitotic rate in primary culture.

In the present study, we attempted in a cell culture system, to determine if mesenchymal cells of Rathke's pouch and its surrounding tissue secrete growth factor(s) for pituitary glandular cells.

Materials and methods. Anterior pituitary clonal cells (2E6) were employed in this study. The 2E6 clonal strain was derived from the NRR5 cell line which was established from Rathke's pouch epithelium of fetal rats of 11–13 days gestation (15). These cells secrete both growth hormone (GH) and prolactin (PRL). Two types of mesenchymal cells were used: (a) a fetal mesenchymal clonal strain which was established from Rathke's pouch and its surrounding tissue from fetal rats of 11–13 days gestation, and (b) a fibroblast strain which was derived from subcutaneous loose connective tissue of 1- to 5-day postnatal rats. These cells were cultured using growth medium consisting of Ham's F10 medium (16) supplemented with 10% fetal calf serum (Gibco, virus screened) and antibiotics (50 U of penicillin and 50 μg of streptomycin/ml, Gibco). All incubations were carried out in a CO_2 incubator at 37° in an atmosphere of 5% CO_2, 5% O_2, and 90% air.

The conditioned medium used in this experiment was taken from cultures of these two types of mesenchymal clonal strains. These cells (1×10^6 cells/dish) were cultured for three days in the above growth medium. The conditioned medium was then diluted to 10%, 20%, and 30% with fresh growth medium. The diluted conditioned medium was then used in the experiment.

Fresh assay media (normal growth medium for control and the various dilutions of conditioned medium for the other dishes) containing ^{3}H-thymidine (7.5 μCi/ml) were added to cultures of 2E6 clonal cells. The cells were incubated in the control or experimental media for 6 hr. After the incubation the 2E6 cells were washed with Eagle's balanced salt solution containing 0.1% BSA, transferred to a sealed vial, and hydrolysed in 0.5 ml of 5% TCA for one hour at 100°. The cell hydrolysate was then mixed with 10 ml of scintillation fluid (8 g Omnifluor, New England Nuclear, and 100 g naphthalene per liter of 1,4 dioxane), and radioactivity per 2E6 cell was estimated by liquid scintillation counting.

After a six hour incubation of 2E6 clonal cells with the conditioned medium from the two strains, the conditioned medium from

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each dish was collected and stored at −20° for radioimmunoassay of GH and PRL. In another experiment the effect of growing 2E6 cells in association with the two types of mesenchymal cells was examined. Mesenchymal cells from Rathke's pouch or skin fibroblasts were cultured in growth medium (1×10^5 cells/4 ml) for 3 days. The conditioned medium was discarded and the cells were added to cultures of 2E6 cells (1×10^6 cells/4 ml) and cultured for 24 hr. The conditioned medium was then replaced with fresh growth medium (4 ml/dish). After 6 hours of incubation in culture medium the conditioned medium was collected and frozen for RIA of GH and PRL.

The amount of PRL contained in the culture medium was measured by the method of Niswender *et al.* (16). GH was assayed by the method of Birge *et al.* (17). The number of living cells which did not stain with 0.1% trypan blue (in BSS) was counted using a Burker–Turk hemocytometer. The cell protein content of the cultures was determined by the method of Lowry *et al.* (19).

Results. In the control culture, using normal growth medium, the cell number increased from 1×10^5 cells/dish to 5×10^6 cells/dish in 13.5 days. When the same number of cells was cultured in medium supplemented with 10% conditioned medium from Rathke's mesenchymal cells their number increased to 5×10^6 cells/dish in 11.4 days. In the culture with 20% conditioned medium, the cell number rose to 5×10^6 cells/dish in 9.8 days. The doubling time of 2E6 cells under the above culture conditions were 2.8, 2.6 and 2.4 days, respectively. This growth rate decreased markedly after the population density reached about 5×10^6 cells but when the cells were diluted to 1×10^5/dish cell growth again showed a marked increase (Fig. 1). However, when the cells were cultured in growth medium supplemented with 10% or 20% of the medium conditioned with skin fibroblasts the number of 2E6 cells did not increase.

The incorporation of ^{3}H-thymidine into 2E6 cells which were incubated for six hours in normal growth medium supplemented with ^{3}H-thymidine and in various dilutions of conditioned medium (10%, 20%, 30%) supplemented with ^{3}H-thymidine is shown in Table I. The 10% and 20% media conditioned with Rathke's pouch mesenchymal cells stimulated DNA synthesis (as reflected by ^{3}H-thymidine incorporation) in the 2E6 clonal cells approximately 9 and 20 times that of the control cultures. However, when 30% conditioned medium was added to the growth medium the DNA synthesis of the 2E6 cells increased only about 2.2 times that of the control value. In addition, none of the various dilutions of the conditioned media from cultures of skin fibroblast stimulated DNA synthesis of 2E6 clonal cells.

Table II shows the amounts of GH and PRL secreted from 2E6 cells (2×10^6 cells/dish) cultured with various dilutions of conditioned medium in which Rathke's mesenchymal cells or skin fibroblast had been cultured. The amounts of GH and PRL secreted after addition of 10% conditioned medium from Rathke's mesenchymal cells increased gradually throughout the duration of the culture period. After 7 days, the amount of GH secreted into the dishes containing 10% conditioned medium of Rathke's mesenchymal cells was about 2.6 times higher than that of the control. Additionally, the amount of PRL secreted into this same medium was approximately 1.6 times the control. In the case of the dishes containing 20% or 30% conditioned medium of Rathke's mesenchymal cells, there was some further increase in the amount of GH and PRL secreted into the medium. However, the amount of PRL secreted in seven days in 20% or 30% conditioned medium was not appreciably greater than that present in the medium after 3 or 5 days incubation. In the case of GH production there was some further increase seen at 5 and 7 days. Throughout the course of the experiment, the 2E6 clonal cells cultured with growth medium supplemented with conditioned medium (10%, 20% and 30%) of skin fibroblast did not exhibit any increase in GH or PRL secretion.

In the cultures of 2E6 and Rathke's mesenchymal cells, the amounts of GH secreted were 1.5, 2, 3, and 3.7 times higher than those of the control at 1, 3, 5, and 7 days, respectively. Similarly, the amounts of PRL secreted were 1.1, 1.4, 1.6 and 2 times higher than those of the control at 1, 3, 5, and 7 days. However, the skin fibroblasts stimulated neither GH nor PRL secretion by the 2E6 cells (Table III).

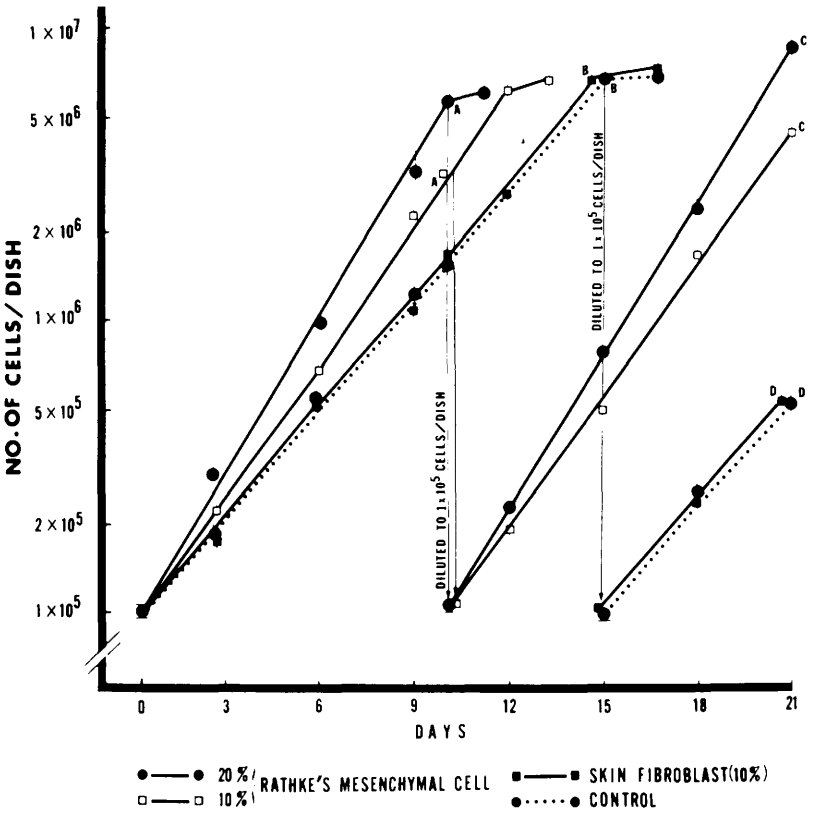

FIG. 1. Cell growth of 2E6 cultured with conditioned medium of Rathke's mesenchymal cells or skin fibroblasts. A: $P < 0.005$; B: not significant; C: $P < 0.005$; D: not significant.

TABLE 1. THE INCORPORATION OF ^{3}H-THYMIDINE INTO 2E6 CELLS.

Treatment	cpm/mg cell protein[a]
Control	1,308
Conditioned medium	
10%	12,057
20%	27,854
30%	2,851
Skin fibroblast	
10%	1,406
20%	1,482
30%	1,577

[a] The value of each group is mean value of three dishes.

For control purposes, skin fibroblasts and Rathke's pouch mesenchymal cells were cultured by themselves for 21 days in normal growth medium and medium supplemented with one μg/ml of rat median eminence extract. Assay of this conditioned medium revealed no detectable amounts of hormones.

Discussion. Both transformed cells and cells in primary cultures have been found to secrete a biologically active nerve growth factor (NGF) that is immunologically similar to the β-NGF isolated from the submandibular glands of the male mouse. Since this study has been performed on various types of cells from several different animals, including man (20), it has been suggested that the secretion of NGF may be a property of many kinds of cells (21).

Rutter's group proved that extracts from mesenchymal tissue stimulated the normal development of rat pancreatic epithelia *in vitro* (2–5). A specific component of embryonic mesenchymal tissue appears to be the cause of the stimulation, since stimulation only occurs in cultures with extracts of tissues rich in mesenchyme and in tissue culture cells derived from mesenchyme (5, 6). Furthermore, Tixer-Vidal and coworkers suggested from their primary cultures of the anterior pituitary gland that the fibroblasts in the anterior pituitary may secrete a factor(s) necessary for the survival of the glandular cells.

TABLE II. EFFECTS OF CONDITIONED MEDIUM OF RATHKE'S POUCH MESENCHYMAL CELLS OR CLONAL FIBROBLASTS ON GH AND PRL RELEASE OF 2E6 CELLS.

Treatment	GH (ng/mg cell prot.)*	PRL (ng/mg cell prot.)*
Control	462 ± 24.3[a,c,e]	1,557 ± 97.5[b,d,g]
Conditioned medium		
Rathke's pouch mesenchymal cell 10%		
1 day	517 ± 27.6**[a]	1,593 ± 83.7
3 days	703 ± 44.2	1,929 ± 148.2[b]
5 days	936 ± 45.9	2,218 ± 172.4
7 days	1,208 ± 83.5[c]	2,373 ± 153.2
Rathke's pouch mesenchymal cell 20%		
1 day	695 ± 42.8[c]	1,899 ± 117.4[d]
3 days	879 ± 42.7	2,438 ± 183.1
5 days	1,109 ± 89.3	3,658 ± 157.4
7 days	1,792 ± 145.1	2,602 ± 120.5
Rathke's pouch mesenchymal cell 30%		
1 day	755 ± 38.1	1,972 ± 147.9
3 days	904 ± 48.3	2,593 ± 186.4
5 days	1,206 ± 84.9	2,699 ± 156.2
7 days	1,873 ± 48.7	2,680 ± 117.4
Skin fibroblast 10%		
1 day	444 ± 36.2[f]	1,548 ± 76.7
3 days	448 ± 27.1[f]	1,502 ± 83.5[g]
5 days	430 ± 17.6	1,561 ± 72.4
7 days	434 ± 23.9	1,530 ± 68.1
Skin fibroblast 20%		
1 day	458 ± 17.6	1,497 ± 78.3
3 days	429 ± 31.8[f]	1,532 ± 81.4[g]
5 days	411 ± 20.5	1,497 ± 76.3
7 days	466 ± 19.5	1,511 ± 83.7
Skin fibroblast 30%		
1 day	433 ± 18.2[f]	1,478 ± 93.1[g]
3 days	428 ± 32.7	1,502 ± 98.5
5 days	441 ± 20.8	1,528 ± 110.4
7 days	455 ± 32.3	1,472 ± 89.6

* Hormone levels released into media were calculated to amount per mg protein of cells which finally existed in the media.
** Mean ± SE.
[a] = $P < 0.1$.
[b] = $P < 0.01$.
[c] = $P < 0.005$.
[d] = $P < 0.01$.
[e] = $P < 0.001$.
[f] = Not significant.
[g] = Not significant.

TABLE III. EFFECTS OF ASSOCIATED RATHKE'S POUCH MESENCHYMAL CELLS OR CLONAL FIBROBLASTS ON GH AND PRL RELEASE OF 2E6 CELLS.

Treatment	GH (ng/mg cell prot.)	PRL (ng/mg cell prot.)
Control	462 ± 24.3	1,557 ± 97.5
Associated culture		
Rathke's pouch mesenchymal cell		
1 day	681 ± 32.3*[a,c,d]	1,724 ± 92.6[b,e]
3 day	908 ± 31.5[a]	2,224 ± 111.5[b]
5 days	1,386 ± 98.1[c]	2,494 ± 104.7
7 days	1,706 ± 120.8[d]	3,113 ± 143.6[e]
Skin fibroblast		
1 day	440 ± 25.8	1,535 ± 81.4
3 days	450 ± 21.7	1,533 ± 79.4
5 days	431 ± 19.7	1,590 ± 91.2
7 days	439 ± 20.9	1,503 ± 114.2

* Mean ± SE.
[a] = $P < 0.005$.
[b] = $P < 0.01$.
[c] = $P < 0.001$.
[d] = $P < 0.0001$.
[e] = $P < 0.001$.

In addition, there is an opinion that follicular cells in the anterior pituitary gland may have similar functions to those of Sertoli cells in the testis. In the anterior pituitary gland of an anencephalic fetus, there are many cells between the glandular cells that are morphologically similar to mesenchyme cells. These mesenchymal-like cells have a number of glycogen granules in their cytoplasm (unpublished observations).

In this study, mesenchymal cells of Rathke's pouch and its surrounding tissue secreted a substance(s) which stimulated not only DNA synthesis of 2E6 clonal cells but also the GH and PRL secretion by these cells *in vitro*. However, median eminence extract stimulated PRL secretion but inhibited GH secretion by 2E6 clonal strain (unpublished observations). These facts imply that the mesenchymal factor(s) may be different than those of median eminence extracts. This mesenchymal factor(s) may also be different from the NGF secreted by L cells, since L cells are skin fibroblasts derived from loose connective tissue, and the skin fibroblasts did not stimulate DNA synthesis and hormonal secretion of the 2E6 clonal strain. Also, we have shown (unpublished observations) that when

Rathke's mesenchymal strain cells were cultured in medium containing median eminence extract and/or fetal brain extract they did not produce any anterior pituitary hormones.

It is still not known why the DNA synthesis of 2E6 cells cultured with growth medium supplemented with 30% conditioned medium of Rathke's mesenchymal cells was decreased markedly. There are two possibilities that may explain this result. One is that some inhibitor is produced by Rathke's mesenchymal cells, and another is that the growth medium may be diluted too much by the 30% conditioned medium.

Numerous papers have been published concerning the autodifferentiation of pituitary cells after encephalectomy (8–13) and organ cultures of pituitary anlage cells (21, 22). However, the encephalectomies in the above experiments were performed at 16 days gestation of the fetal rat, after the pituitary gland has already been formed. At this time, the pituitary anlage cells have already differentiated into committed cells. Therefore, these experiments failed to determine whether or not pituitary anlage cells autodifferentiated after the encephalectomies of the fetal rats. Autodifferentiation of pituitary cells in an organ culture of Rathke's pouch tissue is also difficult to prove because there are many mesenchymal cells in the area of Rathke's pouch. It is still unknown whether a stimulator secreted by Rathke's pouch mesenchymal cells could also be secreted by mesenchymal cells of the anterior pituitary after 15 days gestation.

A possibility exists that cells derived from Rathke's pouch and surrounding tissue secrete unknown factor(s) which stimulate the differentiation of multipotential (uncommitted) cells to committed cells. In this study, progenitor cells of the 2E6 clonal strain could differentiate into GH or PRL secreting cells by the action of this unknown substance(s). Since Rathke's mesenchymal factor(s) is not secreted by skin fibroblasts, this factor's main action may be the differentiation of pituitary anlage cells into committed cells.

However, an important additional action of mesenchymal factors is clearly the stimulation of cell proliferation. It remains for future studies to determine if mesenchymal cells of tissues remote from Rathke's pouch in 11- to 13-day old fetal rats can also secrete factors which increase cell proliferation and/or cell differentiation in cells of the pituitary anlage.

Summary. 2E6 clonal cells were cultured with (a) mesenchymal cells derived from Rathke's pouch and the surrounding tissue from fetal rats of 11–13 days gestation, (b) skin fibroblast cells derived from loose connective tissue under the skin of 1- to 5-day postnatal rats, (c) conditioned medium taken from a culture of these Rathke's mesenchymal cells and diluted to 10%, 20%, and 30% with growth medium, and (d) conditioned medium taken from a culture of skin fibroblast cells and diluted to 10%, 20%, and 30% with growth medium. Radioimmunoassay of the media from these cultures revealed an increase in both GH and PRL secretion in the cultures containing 2E6 cells and Rathke's mesenchymal cells and 2E6 cells grown in conditioned media from the Rathke's mesenchymal cells. However, the cultures of 2E6 and skin fibroblasts or its conditioned media showed no increase in hormonal secretion. Rathke's pouch and surrounding mesenchymal cells and its conditioned media stimulated cell proliferation in the 2E6 clonal cells whereas the skin fibroblasts and their conditioned media did not. We concluded that the mesenchyme of Rathke's pouch and its surrounding tissue secreted a growth factor(s) which stimulated hormonal production and secretion and cell proliferation in pituitary glandular cells.

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