Growth and differentiation of rat egg-cylinders under the kidney capsule

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SUMMARY

Two groups of embryonic shields were transferred under the kidney capsule, on days 7½ and 9 of normal pregnancy. The first group was without mesoderm and the second one with well-formed mesoderm. Random-bred and inbred Fischer rats were used.

Killed and fixed 15 and 30 days after the operation, the grafts looked like teratomata with well-differentiated tissues. Differentiation was the same in embryos explanted at 7½ and 9 days, and from the two strains of rat.

In order to study the growth potential, transfers within and between each strain of rat were carried out. A difference in weight was found 15 days after the operation among some series. This was not observed 30 days after the transfer. Although the grafts in random-bred rats were larger than in the inbred Fischer strain, factors other than the immunological influence were invoked for the explanation of the initial difference in growth.

Long-term grafts were studied only in the inbred strain. Two, 4 and 6 months after the operation all well-differentiated tissues were still present. The amount of growth was very variable within each series, but no graft was found totally resorbed.

Only in the largest graft, 6 months after the operation, large masses of cells were found which seemed to be undifferentiated.

INTRODUCTION

Transfers of tubal mammalian eggs or uterine blastocysts to ectopic sites have been performed several times in order to test their developmental capacities in an extra-uterine environment. In almost all of these experiments only the capacity for early embryonic tissue formation and not that for the histogenesis of fully differentiated tissues was tested (e.g. Fawcett, 1950; Kirby, 1960, 1963a, b, 1965; Porter, 1967; Billington, Graham & McLaren, 1968).

The analysis of final histogenesis in ectopic sites has been carried out relatively rarely. Grobstein (1950a, 1951, 1952a, b) tested the capacity for differentiation of mouse egg-cylinders grafted into the anterior chamber of the eye after various experimental pretreatments. Recently Stevens (1968) obtained in only one specific mouse strain a few well-differentiated tissues following transfer of tubal eggs into the testis.

Nicholas (1942) reported that rat tubal eggs and egg-cylinders at different

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developmental stages, when transplanted to the kidney, gave rise to many disorganized differentiated tissues including typical mesodermal ones. As his results concerning the 2- to 4-cell eggs were not confirmed by recent investigation (Kirby, 1962), his findings concerning the egg-cylinders are open to question.

Rat embryos have so far been grown in vitro for a few days only (Grobstein, 1950; New & Daniel, 1969) which is insufficient for a precise analysis of final histogenesis.

The major object of our interest is the capacity for growth and differentiation of definite tissues in extra-uterine sites from early postimplantation rat embryos. In a previous paper (Levak-Svajger & Skreb, 1965) we have shown that rat egg-cylinders, when transferred into the anterior chamber of the eye before mesoderm formation, usually gave rise solely to ectodermal and endodermal tissues. However, if transplantation took place after mesoderm formation, all three germ layers underwent typical histogenesis and well-differentiated mesodermal tissues (cartilage, bone, muscle) were among those developed from the grafts.

The question arose whether this different histogenetic behaviour resulted from factors intrinsic to embryos at different stages of development or whether it was due to extrinsic influences of a particular extra-uterine environment. In order to establish this we have now tested the growth and histogenetic capacities of rat embryos transplanted under the kidney capsule as an extra-uterine site which is different in several details from the anterior chamber of the eye. In addition, the stability of the histodifferentiation attained was analysed in long-term grafts.

In our previous grafting experiments into the anterior chamber of the eye we used random-bred rats and sometimes found strong immunological reactions. Thus, during this study we used two different strains of rats in order to see whether immunological factors can influence embryo growth and differentiation in an ectopic site.

MATERIAL AND METHODS

Two strains of albino rats were used in this experiment: one having been randomly bred for about 20 years in our laboratories (R), and the other an inbred Fischer strain (F). Gestation was considered to have begun early in the morning when sperm was found in the vaginal smear. The eggs were considered to be 1 day old 24 h after sperm had been found. It was histologically verified that embryos of both strains had still only two germ layers at \( t = 3 \) days, as has been stated previously by other authors (Huber, 1916; Mulnard, 1955). On day 9 the third layer was already formed.

Two groups of females were anaesthetized with ether on gestation days 7 and 9. With watchmaker’s forceps the uterus wall was gently torn open and the entire egg-cylinder (embryonic shield + extra-embryonic part + ectoplacental cone) isolated. The lengths of the 7- and 9-day-old cylinders without the cone
Growth of egg-cylinders in kidney

were in the case of the random-bred rats about 0.50 and 1.50 mm and in Fischer-rats about 0.35 and 1.00 mm.

After the removal of Reichert's membrane and the ectoplacental cone the egg-cylinder was cut transversely at the junction of the embryonic and extra-embryonic parts which is clearly visible even in $7\frac{1}{2}$-day-old cylinders. The extra-embryonic part was discarded and only the embryonic shield was transferred by means of a braking pipette under the kidney capsule of a recipient adult rat about 3 months old (male or female).

On 15, 30, 60, 120 and 180 days following transfer, the animals were killed and both kidneys removed and weighed. The difference in weight between the experimental kidney containing the graft and the contralateral control kidney was taken as a measure of the amount of growth of the graft. In a preliminary study we found that in both strains the right and left kidneys are not always identical in weight, but the difference was not statistically significant.

The grafts with a small amount of the underlying host tissue were fixed in Zenker's fluid, embedded in paraffin wax, serially sectioned and stained with hemalum and eosin. From very large grafts only small pieces of tissue were examined histologically.

Table 1. The differentiated grafts after the transfer beneath the kidney capsule

<table>
<thead>
<tr>
<th>Series</th>
<th>Stage at transfer (days)</th>
<th>Strain*</th>
<th>Experimental period (days)</th>
<th>No. transferred</th>
<th>No. hemor rhagic cysts</th>
<th>No. well-differentiated grafts</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7 $\frac{1}{2}$</td>
<td>F</td>
<td>15</td>
<td>35</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>7 $\frac{1}{2}$</td>
<td>R</td>
<td>15</td>
<td>28</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>F</td>
<td>15</td>
<td>13</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>R</td>
<td>15</td>
<td>10</td>
<td>—</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>F/R</td>
<td>15</td>
<td>14</td>
<td>—</td>
<td>14</td>
</tr>
<tr>
<td>6</td>
<td>9</td>
<td>R/F</td>
<td>15</td>
<td>10</td>
<td>—</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>7 $\frac{1}{2}$</td>
<td>F</td>
<td>30</td>
<td>28</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>8</td>
<td>7 $\frac{1}{2}$</td>
<td>R</td>
<td>30</td>
<td>35</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>9</td>
<td>9</td>
<td>F</td>
<td>30</td>
<td>10</td>
<td>—</td>
<td>10</td>
</tr>
<tr>
<td>10</td>
<td>9</td>
<td>R</td>
<td>30</td>
<td>12</td>
<td>—</td>
<td>12</td>
</tr>
<tr>
<td>11</td>
<td>9</td>
<td>F</td>
<td>60</td>
<td>6</td>
<td>—</td>
<td>6</td>
</tr>
<tr>
<td>12</td>
<td>9</td>
<td>F</td>
<td>120</td>
<td>6</td>
<td>—</td>
<td>6</td>
</tr>
<tr>
<td>13</td>
<td>9</td>
<td>F</td>
<td>180</td>
<td>10</td>
<td>—</td>
<td>10</td>
</tr>
</tbody>
</table>

* F = Fischer strain, R = randomly bred rat, F/R = Fischer embryo transferred to R host, R/F = R embryo transferred to F host.

RESULTS

Table 1 shows that about 50 % of the $7\frac{1}{2}$-day-old embryonic shields survived as grafts. It is uncertain whether the remaining $7\frac{1}{2}$-day-old embryos died after the transfer or whether they were lost during the experimental procedure. The first embryos transferred were not completely devoid of extra-embryonic cells
and developed into hemorrhagic cysts with little or no embryonic tissue. Therefore in subsequent experiments special attention was paid to cutting off any extra-embryonic parts.

From 126 embryos explanted at \( 7\frac{1}{2} \) days we obtained 28 well-differentiated grafts all showing a similar degree of differentiation.

The 9-day-old embryos were easily transferred and almost all gave rise to well-differentiated tissues.

**Differentiation**

15 and 30 days after operation. In all the series from 1 to 10 the successful grafts contained mesodermal tissues, in spite of the fact that embryos of series 1, 2, 7 and 8 had no mesoderm at transfer. Even in the first two series in which embryonic shields without mesoderm were grafted for only 15 days, five of the eight successful grafts contained well-differentiated cartilage. The incidence of tissues found in grafts 30 days after operation (series 7–10) is given in Table 2. The same tissues also developed in series 1–6 but in addition thymus was present in three grafts of series 2, in six grafts of series 4 and in one graft of series 6. In a few grafts, without any correlation with a particular series, thyroid, enamel organ and epithelium of the genital tract were present. Differentiation of liver and kidney was never observed.

Table 2. Tissues found in grafts 30 days after operation

<table>
<thead>
<tr>
<th>Series</th>
<th>Stage at transfer (days)</th>
<th>Strain*</th>
<th>No. well-differentiated grafts</th>
<th>Neural tissue</th>
<th>Epithelium</th>
<th>Cartilage</th>
<th>Bone</th>
<th>Adipose tissue</th>
<th>Skeletal muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>7(\frac{1}{2})</td>
<td>F</td>
<td>8</td>
<td>7</td>
<td>4</td>
<td>7</td>
<td>6</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>7(\frac{1}{2})</td>
<td>R</td>
<td>12</td>
<td>8</td>
<td>6</td>
<td>10</td>
<td>8</td>
<td>11</td>
<td>7</td>
</tr>
<tr>
<td>9</td>
<td>9</td>
<td>F</td>
<td>10</td>
<td>8</td>
<td>6</td>
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<td>9</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>10</td>
<td>9</td>
<td>R</td>
<td>12</td>
<td>12</td>
<td>11</td>
<td>10</td>
<td>6</td>
<td>12</td>
<td>5</td>
</tr>
</tbody>
</table>

* F = Fischer strain, R = randomly bred rat.

The results listed in Table 2 clearly demonstrate that the rat embryonic shield even at the two-layer stage can give rise to well-differentiated mesodermal tissues if grafted under the kidney capsule (Fig. 1A, B). At this stage the kidney grafts differ strikingly from eye grafts of the same embryonic stage, in which mesodermal tissues differentiated significantly less frequently (Levak-Švajger & Škreb, 1965). As far as differentiation is concerned the two different strains (R, F) do not differ from each other except for the round cell infiltration which occurred in some R grafts after 30 days (Fig. 1B).

2, 4 and 6 months after the operation. In the last three series (11–13, inbred Fischer strain) only one small piece of each graft was examined histologically. In spite of that the majority of tissues mentioned before were found even after 6 months of development (Fig. 1C). Even brain and ganglion were found
Fig. 1. Sections of different grafts. (A) 7½-day-old egg-cylinder after 30 days under the kidney capsule. Various well-differentiated tissues: bone, cartilage, brain, glands etc. Fischer strain. (B) 7½-day-old egg-cylinder after 30 days under the kidney capsule. Cartilage and muscle are present. Note round cell infiltration. Random-bred rat. (C) 9-day-old egg-cylinder 4 months after the operation. Muscle and adipose tissue are present and at the bottom brain tissue. Fischer strain. (D) 9-day-old egg-cylinder 6 months after the operation. Graft No. FT 17. Note cluster of undifferentiated cells.
without any sign of necrosis. Only in the largest graft (about 31 g) from series 13, large necrotic centres were observed in an apparently undifferentiated tissue (Figs. 1D, 2).

**Hemorrhagic cysts**

Some of the 7½-day-old embryos only developed into hemorrhagic cysts with very few embryonic cells and large masses of host blood. The same result was obtained when mouse tubal eggs (e.g. Fawcett, 1950; Kirby, 1965; Porter, 1967), mouse egg-cylinders with their cones (Kirby, 1963b) or mouse ecto-placental cone alone were transferred to ectopic sites (Billington, 1965).

![Photograph of the largest graft No. FT 17, 6 months after operation. Note the size of the graft in comparison with the contralateral kidney.](image)

**Growth**

In Table 3, only differentiated grafts were included.

In series 3–6 (9-day-old embryo, 15 days after operation) the weight within each series was very similar, so that the standard error was relatively low. There were, however, some differences between series, the random-bred embryos growing better than the Fischer embryos (series 3, 4). In order to ascertain the cause of this difference, we made some transfers between the two strains (series 5, 6). It may be concluded that the Fischer embryo grew better in a random-bred host than in a Fischer host (series 3, 5). However, the increase in weight of the random-bred graft was not significantly different in the Fischer and in the random-bred host (series 4, 6). If the two kinds of graft are compared in the Fischer host, the random-bred grew better than the Fischer embryos (series 3, 6).
The weight of kidneys can vary from one series to another (e.g. series 3, 4). To evaluate a possible correlation between the weight of the kidney and its graft, the coefficient of correlation was calculated for series 3–6 (44 cases) and the value $r$ which was obtained (0-2556) did not even approach statistical significance at the 5% probability level.

**Table 3. The weights of grafts**

<table>
<thead>
<tr>
<th>Series</th>
<th>Stage at transfer (days)</th>
<th>Strain*</th>
<th>Experimental period (days)</th>
<th>No. successful grafts</th>
<th>Weight (mg)</th>
<th>Weight of contralateral kidney (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$7_\frac{1}{2}$</td>
<td>F</td>
<td>15</td>
<td>5</td>
<td>26 ± 12†</td>
<td>541 ± 9</td>
</tr>
<tr>
<td>2</td>
<td>$7_\frac{1}{2}$</td>
<td>R</td>
<td>15</td>
<td>3</td>
<td>66 ± 15</td>
<td>624 ± 28</td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>F</td>
<td>15</td>
<td>10</td>
<td>31 ± 6</td>
<td>574 ± 20</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>R</td>
<td>15</td>
<td>10</td>
<td>135 ± 20</td>
<td>885 ± 30</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>F/R</td>
<td>15</td>
<td>14</td>
<td>73 ± 7</td>
<td>721 ± 20</td>
</tr>
<tr>
<td>6</td>
<td>9</td>
<td>R/F</td>
<td>15</td>
<td>10</td>
<td>95 ± 15</td>
<td>716 ± 30</td>
</tr>
<tr>
<td>7</td>
<td>$7_\frac{1}{2}$</td>
<td>F</td>
<td>30</td>
<td>8</td>
<td>506 ± 300</td>
<td>624 ± 28</td>
</tr>
<tr>
<td>8</td>
<td>$7_\frac{1}{2}$</td>
<td>R</td>
<td>30</td>
<td>12</td>
<td>753 ± 216</td>
<td>907 ± 50</td>
</tr>
<tr>
<td>9</td>
<td>9</td>
<td>F</td>
<td>30</td>
<td>10</td>
<td>460 ± 220</td>
<td>806 ± 10</td>
</tr>
<tr>
<td>10</td>
<td>9</td>
<td>R</td>
<td>30</td>
<td>12</td>
<td>444 ± 74</td>
<td>876 ± 30</td>
</tr>
<tr>
<td>11</td>
<td>9</td>
<td>F</td>
<td>60</td>
<td>6</td>
<td>1587 ± 690</td>
<td>965 ± 20</td>
</tr>
<tr>
<td>12</td>
<td>9</td>
<td>F</td>
<td>120</td>
<td>6</td>
<td>3374 ± 950</td>
<td>1032 ± 45</td>
</tr>
<tr>
<td>13</td>
<td>9</td>
<td>F</td>
<td>180</td>
<td>10</td>
<td>9208 ± 3080</td>
<td>1271 ± 50</td>
</tr>
</tbody>
</table>

* F = Fischer strain, R = randomly bred rat, F/R = Fischer embryo transferred to R host, R/F = R embryo transferred to F host.
† Standard error of the mean. Difference between series 3 and 5, $t = 3-59$, $P < 0-01$, between series 4 and 6, $t = 1-59$, $P < 0-1$.

In series 7–10 there were no differences between the series, but the variability was greater within each series. The weight of the largest graft was about 2 g.

This variability increased in series 11–13. The range of weight was in these series as follows: 640–5071, 550–6165 and 938–31328 mg. Thus it seems that some grafts continued growing 4–6 months after the operation.

**DISCUSSION**

(a) Our results clearly indicate that the rat embryonic shield with only two germ layers, when transferred under the kidney capsule has the same differentiative capacity as one with three layers. These data are at variance with our transfer experiments of the same stages into the anterior chamber of the eye, but confirm the findings of Nicholas (1942). However, Nicholas says that in his egg-cylinders without mesoderm 'the membranes were kept intact'. In our embryos when some extra-embryonic cells were left on the graft, only hemorrhagic cysts without embryonic differentiation were obtained. Also, contrary to Nicholas' observations, our long-term grafts were not resorbed.

Stevens (1968) obtained some well-differentiated tissues following trans-
plantation of tubal mouse eggs to the testis, but he used a specific strain which had a very high incidence of spontaneous testicular teratomata. All other mouse strains used in his experiments did not support the development of tubal ova in the testis. Therefore these findings have a limited significance and cannot be compared with ours. In his grafts many groups of undifferentiated cells were found from 15 to 60 days after the operation: this was never observed in our grafts 30 days after the operation, although in one extremely large graft, we found, after 6 months, groups of cells which seemed to be undifferentiated (Fig. 1 D).

If we now try to explain the difference between our previous results with eye grafts (Levak-Švajger & Škreb, 1965) and the data presented in this communication, we can formulate a hypothesis of the impact of the external environment on differentiation of the 2-layered young rat embryo. In the anterior chamber of the eye, the embryo must first penetrate into the surrounding tissues before it is capable of differentiation. Probably the young embryo is more sensitive to this process of penetration and vascularization than an older one. Grobstein’s (1950b, 1951) results tend to favour this idea. In vitro culture, before implantation into the eye, or in vitro culture on plasma clot alone, do not permit the young embryonic shields without head fold to differentiate as well as the older ones. Furthermore, heterografts of rat embryos in the large anterior chamber of the rabbit eye having no contact with the host’s tissue, float in the aqueous humour and survive but show no differentiation at all (Škreb & Levak, 1960). Immediately after transfer under the kidney capsule, however, the embryo lies between the capsule and the kidney parenchyma, both very well vascularized. Blood capillaries probably bud into the graft from both sides, the graft being thus vascularized immediately without an initial period of incorporation into the host tissue. The connexion between the host and graft tissues remains only vascular. These differences in the initial behaviour of grafts in two different ectopic sites could probably be considered as an explanation for differences in their capacities for differentiation.

(b) It is well known that during normal implantation (James, 1965; McLaren, 1965; Kirby, 1967) and also during the development of blastocysts in ectopic sites (Kirby, Billington & James, 1966; Kirby, 1968), an immunological reaction takes place. Furthermore, in xenogenic grafts of rat embryo in the rabbit eye, no differentiation was observed without application of immunosuppressive factors and the well-implanted grafts were quickly resorbed (Škreb & Levak, 1960). It is possible that the degeneration, regression and final resorption in Nicholas’ experiments (1942) were due to the same immunological reaction.

Are the differences in the amount of growth of the grafts (e.g. series 3-6) due to immunological factors? James (1965) found that antigenic dissimilarity between mother and foetus evokes a reaction which influences placental size. Billington (1965) claimed that ectoplacental cones transplanted to genetically dissimilar hosts' testes produced a greater increase in testis weight. In spite of
these data our findings cannot be explained by immunological factors. If F
grafts grow more in R than in F hosts, why do R grafts not grow better in F than
in R hosts? The R embryos were larger at transfer than the F embryos and it is
possible that the R kidney provides a more favourable environment than does
the F kidney, but there is no evidence of an immunological effect. Clarke’s
findings (1969) differ from those of Billington, and her ‘results are interpreted
as suggesting that factors other than immunological influences are determining
the growth of 7½-day-old ectoplacental cones transplanted to the testis’.

In our experiments grafts between random-bred rats did not provoke a
strong immunological reaction and, apart from some round cell infiltration, did
not significantly differ at 30 days from grafts between rats of the inbred strain.
After 30 days wide variations appear in the size of grafts within each series,
but the reasons for this are as yet unknown.

RESUME

Croissance et differentiation des cylindres-oeufs du rat sous la capsule renale

Deux groupes de cylindre-oeufs de rats ont ete greffes sous la capsule renale a 7 jours et
9 jours de la gestation normale. Le premier groupe ne possedait pas encore de mésoderme
tandis que le second en avait deja tres bien forme. On a utilise deux colonies de rats: une
maintenue par accouplement au hassard et l’autre de la souche Fischer.

Les animaux sacrifices 15 et 30 jours apres l’operation presentaient des greffes qui ressem-
blaient a des teratomes et contenaient differents tissus bien differencies. Aucune difference
n’a ete observee entre les deux groupes ou les deux colonies, de sorte que meme les embryons
transferees sans mésoderme montraient tous les tissus mésodermiques.

Afin d’étudier la capacite de croissance, des greffes a l’interieur d’une meme souche ou
entre deux souches differentes ont ete realises. Les differences de poids constatees entre
quelques series 15 jours apres l’operation ne sont plus visibles ou bout de 30 jours. Bien que les
greffes aient ete plus volumineuses dans la colonie panmictique que dans la souche Fischer,
des facteurs autres que l’influence immunologique ont ete evokes pour expliquer les dif-
ferences initiales dans la croissance.

Les greffes de longue duree ont ete etudies seulement dans la souche Fischer, 2, 4 et 6 mois
apres l’operation, et tous les tissus bien differencies etaient encore presents. Le degre de
croissance etait variable dans chaque serie mais aucune greffe n’etait totalement resorbee.
C’est seulement dans la greffe la plus volumeuse qu’on a trouve, 6 mois apres l’operation,
de grands amas cellulaires qui paraissaient indifferencies.

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