Aggregation between teratocarcinoma cells and preimplantation mouse embryos

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SUMMARY

The ability of two embryonal carcinoma (EC) cell lines, F9 and PCI3, to aggregate with preimplantation 8-cell mouse embryos is described. Both adhere to the embryonic cells and subsequently compact with the embryos. The aggregates form blastocysts in culture. The blastocysts sometimes contain the EC cells, located almost always in their inner cell mass. Differentiated derivatives of EC cells, namely PYS-1 and PYS-2, as well as STO fibroblasts do not aggregate with embryos.

INTRODUCTION

Understanding the nature of cell interactions in mammalian embryos is often hampered by their tiny size. Recently, teratocarcinomas have attracted considerable interest since they may be grown in large quantities in vitro and thus offer a means to overcoming this problem.

Teratocarcinomas can arise spontaneously in the gonads. In the mouse they can also be induced by the transfer of embryos or genital ridges to extra uterine sites (for reviews see Stevens, 1967; Solter, Adams, Damjanov & Koprowski, 1975; Graham, 1977). Typically the tumours contain a wide range of undifferentiated tissues as well as a rapidly dividing undifferentiated stem-cell population called embryonal carcinoma (EC) cells. The EC cells are malignant but under a variety of conditions they can also give rise to differentiated derivatives (Kleinsmith & Pierce, 1964). When EC cells are introduced into a blastocyst by microinjection, they colonize the tissues of the developing embryo (Brinster, 1974, 1975; Mintz, Illmensee & Gearhart, 1975; Mintz & Illmensee, 1975; Papaioannou, McBurney, Gardner & Evans, 1975). These experiments demonstrate that EC cells can interact with the embryo and form morphologically normal tissues.

In this paper I wish to describe an interaction between teratocarcinoma cells and 8-cell preimplantation mouse embryos. I have investigated the ability of two EC cell lines and two differentiated derivatives of another EC cell line to adhere to and aggregate with preimplantation embryos. The distribution
of the progeny of the embryonic and the teratocarcinoma cells in the blastocysts which were formed, is also described.

**MATERIALS AND METHODS**

**The supply of embryos**

The embryos were obtained from natural matings and were (129/Sv × A₂G)F₂ and (C57BL6 × CBA)F₂. They were dissected from the oviducts on the morning of the third day of pregnancy (day of plug = 1st day of pregnancy). At this time the embryos were at the early 8-cell stage.

**The culture and labelling of embryos**

To distinguish between embryonic and teratocarcinoma cells in histological sections of the aggregates, the embryos were labelled with [³H] thymidine using the conditions described by Kelly & Rossant (1976). The embryonic cells were labelled, because the labelling conditions were shown by Kelly and Rossant, to not reduce blastocyst cell number or prevent labelled cells from forming live postimplantation embryos, whereas similar conditions that will label EC cells without influencing their behaviour are not yet known. Thus, briefly, the embryos to be labelled were transferred to microdrops of prewarmed Whitten’s medium (1971) under paraffin oil (Boots Pure Drug Co. U.K., selected for absence of toxicity to cultured embryos). The Whittens medium was supplemented with [³H]thymidine at a concentration of 0-25 μCi/ml (specific activity 47 Ci/mmol. Radiochemical Centre, Amersham, U.K.). The embryos were cultured in the medium for 2 h in a humidified gas mixture of 5 % CO₂, 5 % O₂ and 90 % N₂ at 37 °C. After labelling the embryos were washed three times in Whitten’s medium containing 50 % (v/v) foetal calf serum (FCS) that had been heat inactivated at 56 °C for 30 min. The zona pellucidae were removed by briefly incubating the embryos in prewarmed acidified Tyrode’s solution (Handyside, 1978). They were then returned to fresh Whitten’s medium + 50 % FCS (v/v) and cultured for a further 5 h, the medium being changed every 30 min to dilute out the unincorporated label.

**Cell culture**

The cell lines used were PC13-5, F9-41, PYS-1, PYS-2 and STO fibroblasts. PC13-5 and F9-41 are embryonal carcinoma cells derived from the teratocarcinoma OTT6050 (Bernstine, Hooper, Grandchamp & Ephrussi, 1973). These lines were recloned in this laboratory before use (Adamson, Gaunt & Graham, 1979). Both were grown on tissue culture dishes (Sterilin Ltd., Richmond, Surrey, U.K.) coated with gelatin before use. PYS-1 and PYS-2 were gifts from Drs B. Hogan and C. Babinet respectively. They are both differentiated derivatives from OTT6050 embryoid bodies (Lehman, Speers, Swartzen- druber & Pierce, 1974). STO fibroblasts (Martin & Evans, 1974) were a gift
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from Dr S. J. Gaunt. All the cells were cultured in medium lacking nucleosides and deoxynucleosides (Flow Laboratories, Irvine, Scotland) (Stanners, Eliceiri & Green, 1971) but supplemented with 10% FCS (v/v), at 37°C in a 10% CO₂ in air mixture.

Small lumps of cells for aggregation with the embryos were prepared by trypsinising the cells off the dish and resuspending them at a density of $1 \times 10^6$/4 ml of medium in a 50 mm bacteriological dish (Sterilin). This procedure was performed the evening before an experiment, so that by the following morning the cells had formed small aggregates in suspension (Sherman & Miller, 1978).

The aggregation procedure

After labelling and washing, pairs of 8-cell embryos were placed in prewarmed microdrops of Whitten’s medium supplemented with 10% heat-inactivated FCS (v/v), the drops having been pipetted onto a bacteriological dish and covered with paraffin oil. A lump of cells equivalent in size to one 8-cell embryo blastomere was picked from the bacteriological dish using a mouth-controlled micropipette. A lump of EC cells this size consisted, on average, of an aggregate of eight to nine cells. One lump was placed into each microdrop containing a pair of embryos. Using a finer mouth-controlled micropipette the lump was sandwiched between the two embryos, so that the cultured cells were surrounded by embryonic cells. This arrangement was carefully checked to ensure that the two embryos were each touching the lump of cells. The culture dish was returned to the incubator. Thirty minutes later the microdrop was inspected to check that both embryos were still touching the cells. If the cells and embryos were still sticking to each other, judged by gently pipetting the entire group within the microdrop, they were then returned to the incubator and cultured for a further 24–36 h before being fixed and embedded for sectioning. Both the PYS cell lumps and STO cell lumps, which were also equivalent in size to an 8-cell embryo blastomere, were surrounded by two embryos per lump and cultured under identical conditions. The controls consisted of aggregating two 8-cell embryos together without any cultured cells. These ‘double’ embryos were used to ensure that all the embryonic nuclei were labelled.

Fixation and embedding of the blastocysts and teratocarcinoma cell aggregates

Once the teratocarcinoma cell ↔ embryo aggregates had formed blastocysts they were fixed using Heidenhain’s fixative (Tarkowski & Wroblewska, 1967). Following fixation the blastocysts were placed in a drop of 1% (w/v) molten agar, the agar having been dissolved in a 0.9% (w/v) NaCl solution. When the agar had set, a small block containing the blastocysts (the blastocysts being easily visible in the transparent agar), was cut out and treated as a single
specimen for dehydration and embedding using standard conditions (Hillman, Sherman & Graham, 1972).

The blastocysts were sectioned at 6 μm, stained using Mayers–haemalum and light green, dehydrated, cleared and mounted for examination. The distribution of the nuclei within the blastocysts was drawn using a camera lucida attachment to a Zeiss R.A. microscope.

**Autoradiography**

After sections were drawn, the coverslips were removed by overnight soaking in toluene. The sections were washed thoroughly in ethanol and rinsed in distilled water. They were then washed in two changes of 5 % (w/v) aqueous solution of trichloroacetic acid at 4 °C for 30 min each, washed for 2 h under running tap water, rinsed in distilled water and dipped in Ilford K2 emulsion (Rogers, 1973). The slides were exposed for two weeks, developed in Kodak D19 developer, fixed with Super Amfix (Colay & Baker, Dagenham U.K.) and restained with Mayers–Haemalum and light green. The distribution of labelled nuclei was then marked on the previously made drawings.

The following procedure was used to ensure that the [3H]thymidine used to label the embryonic cells was confined to these cells and that it was not transferred to the teratocarcinoma cells aggregated with the embryos. The EC -> labelled embryo aggregates were incubated in Whitten's medium with 0.02 μg/ml Colcemid (Flow Laboratories) for 4 h (Tarkowski & Rossant, 1976) on the morning of the 4th day of gestation. Chromosome spreads were made by swelling the cells in a 1 % (w/v) aqueous solution of sodium citrate, pH 8.0 for 2 min. The aggregates were transferred to a microscope slide and excess solution removed by pipetting. The aggregates were fixed by dropping a 3:1 ethanol:acetic acid fixative onto them (Tarkowski, 1966) and then dispersed by pipetting a microdrop of 60 % acetic acid onto them. The slides were air dried and stained with toluidene blue. This procedure was performed with the F9 EC cells since they carry chromosome markers that distinguish their karyotype from that of the embryonic cells. The chromosomes were photographed, washed, dipped in K2 emulsion and exposed for two weeks. They were then developed, restained and re-photographed to record which karyotypes were labelled.

**RESULTS**

The results are divided into two sections. The first section describes the observations made on the sticking and subsequent aggregation between the embryo and the cultured cell lumps. The second section deals with the description of the embryo and teratocarcinoma cells' progeny in blastocysts derived from the aggregates.
Fig. 1 (a–f). These Figures illustrate the course of aggregation between a lump of F9 cells (arrowed) and two 8-cell embryos. The times at which the stages were photographed are given: (a) 0 h; (b) 30 min; (c) 1½ h; (d) 3 h; (e) 12 h; (f) 24 h.
Table 1. The ability of cultured cells to aggregate successfully with embryos

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Nos. of aggregation attempted between the cultured cells and embryos</th>
<th>Nos forming blastocysts with no cultured cells visible on the outside of the trophoderm</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>F9</td>
<td>21</td>
<td>19</td>
<td>90</td>
</tr>
<tr>
<td>PCI 3</td>
<td>22</td>
<td>12</td>
<td>55</td>
</tr>
<tr>
<td>PYS-1</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PYS-2</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>STO fibroblasts</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Controls-2 labelled embryos aggregated together</td>
<td>11</td>
<td>9*</td>
<td>82</td>
</tr>
</tbody>
</table>

* These were the number of single blastocysts obtained from the aggregation of two 8-cell embryos.

The sticking and aggregation between the teratocarcinoma cells and embryos

Sticking and aggregation only occurred between the embryonal carcinoma (EC) cells and the embryos. Neither PYS-1, PYS-2 nor the STO fibroblasts aggregated with the embryos.

All the EC cell lumps initially stuck to the embryos and usually this occurred during the first 10 min after having come in contact with each other. Within 5–8 h after starting, the embryos and the EC cells had compacted together to from a single aggregate (Fig. 1). Continued culture of this aggregate resulted in a single blastocyst being formed from a combination of the embryos and the EC cells. Blastocoel formation had begun in all the embryo ↔ EC cell

Fig. 2. An unsuccessfully aggregated PCI 3 EC ↔ embryo blastocyst. The lump of cells (arrowed) attached to the trophoderm is the PCI 3 EC cells.

Fig. 3. An unsuccessfully aggregated PYS-2 ↔ embryo blastocyst. The lump of cells (arrowed) attached to the trophoderm is the PYS-2 cells. These cells form less compacted lumps than EC cells, when in suspension and the cells have multiplied in number over the period of co-culture with the embryos.

Fig. 4. A 6 μm section of an unsuccessfully aggregated PCI 3 EC ↔ embryo blastocyst. The unlabelled PCI 3 EC cells (arrowed) are attached to the outside of the labelled trophoderm.

Fig. 5. An autoradiographed 6 μm section from a successfully aggregated F9 EC ↔ embryo blastocyst. The unlabelled F9 cells are adhering to the inside of the labelled trophoderm.

Fig. 6. An autoradiographed section from a control blastocyst derived from two aggregated embryos. Note all the nuclei are labelled.
aggregates and the two-embryo-aggregate controls by the end of the fourth
day of development.

The frequency of successful aggregation with the incorporation of the EC
cells into the blastocyst differed between the two EC cell lines used. Here,
successful aggregation was defined by the absence of any EC cells either
sticking to the outside of the blastocyst or present within the microdrop, but
unattached to the blastocyst. Ninety percent of the aggregations attempted with
F9 EC lumps were successful, while PC13 successfully aggregated and formed
single blastocysts with the two embryos at a frequency of 55% (Table 1).

The unsuccessful aggregates consisted either of a single blastocyst derived
from both embryos or two blastocysts loosely adhering to each other. In both
situations a lump of EC cells was seen sticking to the outside of the trophecto-
derm of one of the blastocysts (Fig. 2).

The parietal yolk-sac endoderm cell lines PYS-1 and PYS-2 as well as the
STO fibroblasts did not aggregate with the embryos. In all the experiments
it proved to be very difficult to get the PYS and STO cell lumps to remain
sticking to the embryos. When this was achieved in 50% of the aggregations
performed, all the three cell types were easily separated from the embryos by
gentle pipetting, even after having been in continuous contact for 6 h with
the embryos.

All the embryos from the PYS, STO experiments developed to form blasto-
cysts either derived from the two embryos that had aggregated or from single
embryos that had not. These blastocysts had groups of cells, with the morpho-
logy of PYS or STO cells, adhering to the outside of the trophectoderm
(Fig. 3). Though these observations were limited by the resolving power of the
Wild dissecting microscope, used for the manipulations, they were subsequently
supported by the autoradiographic analysis.

The viability of EC cells in the successful EC ↔ embryo aggregate blastocysts
was verified by allowing ten unlabelled blastocysts to attach and outgrow on
tissue culture dishes. Six survived and from all these cultures F9 EC cells
were observed to be growing in the cultures. Giant cells characteristic of
trophectoderm could also be seen within 3 days of the embryos attaching.
Chromosome spreads were made from these cultures and the chromosome
markers characteristic of F9 were found in preparations from all six cultures
(five to six metaphases scored per culture). Similarly, the lumps from three
unsuccessfully aggregated PC13 ↔ embryo blastocysts were removed and
allowed to attach to tissue-culture dishes. All three produced colonies of
EC cells. The viability of the PYS and STO cells was also verified using the
same method and again the cell lumps from three of each unsuccessful aggre-
gation combination, produced colonies, with the characteristic morphologies
of PYS and STO cells.
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Autoradiography of the teratocarcinoma ⩭ embryo aggregate blastocysts

Since EC cells aggregated with the 8-cell embryos, autoradiography was used to look at the distribution of the EC and embryo cells progeny in the blastocysts that had developed from the aggregates. To ensure that no label could be transferred from the labelled embryonic cells to the EC cells aggregated with the embryos, chromosome spreads were prepared from F9 ⩭ labelled-embryo aggregates on the morning of the 4th day of development. The F9 karyotype has a mean of 40 chromosomes. It contains two marker chromosomes; a metacentric number eight derived by centric fusion of the two chromosomes and a chromosome consisting largely of chromosome four with a translocation from either chromosome seventeen or eighteen. Both chromosomes are easily recognisable in preparations (the observations were made on at least 50 spreads by Dr E. P. Evans). Eight aggregates were treated and from these a total of ten positive identifications of the normal karyotype of the embryonic cell were made from seven of the aggregates and photographed. A total of fourteen F9 karyotypes were also identified from all eight EC ⩭ embryo aggregates and these were also photographed. The slides were autoradiographed and the same karyotypes again identified. In all the preparations, the normal karyotypes from the embryonic cells were labelled and the F9 karyotypes were unlabelled (Fig. 7). Seven F9 ⩭ embryo blastocysts, seven PC13 ⩭ embryo blastocysts, three PYS ⩭ embryo blastocysts and six control blastocysts were satisfactorily sectioned and autoradiographed.

All the nuclei from the four labelled control blastocysts were heavily labelled (Fig. 6), whereas none of the nuclei from the two unlabelled control blastocysts contained grains. Thus the labelling conditions used were satisfactory in marking all of the embryonic cells after development to the blastocyst.

In the F9 ⩭ embryo blastocysts the unlabelled EC cells were seen as a discrete group, attached to the inner surface of the trophectoderm and usually associated with the labelled ICM (Fig. 5). In all of the blastocysts the F9 cells were completely enclosed by the labelled trophectoderm, except on their blastocoelic side. No cells that could be unequivocally identified as EC cells were seen to contribute to the trophectoderm.

Four of the seven PC13 ⩭ embryo blastocysts contained unlabelled EC cells enclosed by labelled trophectoderm. The distribution of the PC13 cells did however differ from that of the F9 EC cells. Whereas F9 cells remained as a discrete group, the PC13 cells exhibited a more widespread distribution within the blastocyst. They either formed isolated groups of cells scattered around the inner surface of the trophectoderm or they grew as a single-layered sheet around the trophectoderm. The remaining three PC13 ⩭ embryo aggregates consisted of blastocysts with a lump of cells attached to the outside of the trophectoderm. Serial sectioning of these blastocysts showed that the lumps consisted of unlabelled PC13 EC cells (Fig. 4). Two of the blastocysts were
Fig. 7. This shows the F9 and embryonic karyotypes present in the same preparation. The individual karyotypes are reproduced at a higher magnification. (a, b) is the F9 karyotype before and after autoradiography. Note the absence of any grains over the chromosomes in (b). The two distinctive chromosomes of the F9 karyotype are arrowed; the metacentric chromosome derived by centric fusion of both the no. 8 chromosomes and the 'marker chromosome' derived from a translocation of part of Chr. 17 or 18 to Chr. 4. (c, d) shows the embryonic karyotype before and after autoradiography. The embryonic chromosomes (d) are heavily labelled. Compare with the F9 chromosomes in (b).
Fig. 8. This figure shows a complete series of sections from a successfully aggregated F9 EC ↔ embryo blastocyst. The labelled embryonic nuclei are shaded and the unlabelled EC cell nuclei are unshaded. Note that there are only five (arrowed) embryonic nuclei that could possibly be in the ICM of this blastocyst.

morphologically normal with an inner cell mass and an intact trophectoderm. However, in the third blastocyst the PC 13 cells had traversed the trophectoderm and unlabelled cells, continuous with those outside the blastocysts, were adhering to the blastocoelic surface of the trophectoderm.

All of the autoradiographed EC ↔ embryo blastocysts contained labelled cells present on the inside surface of the labelled trophectoderm (Fig. 8). These
cells were intermingled with the unlabelled embryonal carcinoma cells and thus they were presumed to be inner cell mass cells recruited from the two 8-cell embryos. Five of the blastocysts containing F9 cells were fixed simultaneously with four of the controls. These five EC→embryo blastocysts contained 2, 5, 7, 8 and 13 labelled cells as an integral part of a single inner cell mass along with 28, 52, 49, 33 and 40 unlabelled EC cells respectively. The four control blastocysts, each derived from two aggregated embryos contained a range of between 21 and 30 labelled ICM cells (Fig. 6). Thus, this preliminary evidence suggests that the presence of the EC cells inside the blastocysts correlated with a reduction in the numbers of ICM cells recruited from the embryos. No results, for comparison, were available for the PC13→EC blastocysts.

Four blastocysts from the PYS→embryo aggregates were autoradiographed. In all four, the blastocyst nuclei were labelled whereas the nuclei of the cells attached to the outside of the blastocyst were unlabelled. Thus these findings confirmed the previous observations that the unlabelled PYS did not aggregate with the embryos. The STO→embryo blastocysts were not autoradiographed because no STO fibroblast lumps remained adhering to the embryos prior to fixation.

DISCUSSION

This study has shown that EC cells can adhere to and aggregate with 8- to 16-cell mouse embryos. These aggregates go on to form blastocysts, within which the EC cells are located almost always in the inner cell mass, though this location may have been influenced by the arrangement of the embryos and EC cells at the beginning of aggregation (Hillman et al. 1972).

STO fibroblasts and differentiated derivatives of EC cells, such as PYS-1 and PYS-2, when prepared and aggregated under identical conditions to the EC cells, do not stick or aggregate with the embryos, thus suggesting that there is selectivity in aggregation between cultured cells and preimplantation embryos.

Though aggregation between only two EC cell lines and embryos has been described here, other EC cell lines have also been aggregated with embryos. In an independent study, PSA-1 cells after being surrounded by 8-cell stage blastomeres, were detected in the inner cell masses isolated from the resulting blastocysts (Fujii & Martin, 1979).

However, it has emerged from this study that differences occur between EC cells in their ability to aggregate successfully with the embryos. It is not known why a nullipotent cell, such as F9, is more successful at aggregation than a pluripotent cell such as PC13. Possibly many of the PC13 cells are in a state intermediate between that of the ‘true’ EC cell and that of a differentiated derivative, and thus the PC13 cell could have different adhesive characteristics to those of F9.
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