SURVIVAL OF CELLS IMPLANTED IN THE EMBRYONIC CHICK LIMB BUD:
A DIFFERENCE BETWEEN NORMAL AND MALIGNANT RAT BRAIN CELLS

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SUMMARY

Cells from normal rat brain tissue did not survive and few cells could be found 1 d after grafting. In contrast, cells from a glioma and a carcinogen-treated rat brain survived well and many mitoses were observed. These malignant cells also invaded the limb. The behaviour of normal and malignant cells was followed at shorter times after grafting and some invasion by the normal cells was detected. The first signs of degeneration of normal cells were apparent around 7 h after grafting, and after this the grafts progressively deteriorated. These results support the idea that the ability of cells to survive and grow in embryonic tissues is a characteristic of malignant cells. The findings are discussed in relation to mechanisms of tumour formation.

INTRODUCTION

There has been considerable interest in defining those characteristics which distinguish normal from malignant cells (Sanford, 1974). This has been both from the diagnostic viewpoint of providing tests for malignancy, and also because one might thus obtain insights into the mechanisms involved in malignant change. While investigating the invasive behaviour of a variety of different cells in the developing chick wing bud, we noticed a marked difference in the survival of cells from ‘normal’ lines compared to those from virally transformed derivatives (Tickle, Crawley & Goodman, 1978 a). The ‘normal’ cells, BHK (Stoker & Macpherson, 1964) and Nil 8 (Critchley, Chandrabose, Graham & Macpherson, 1974), did not survive as well as the transformed cells, PyBHK and HSV Nil 8. In addition, we found rather surprisingly that the normal cells as well as the transformed cells showed invasive behaviour. We were keen, therefore, to extend these observations to other cells which had been characterized in a number of ways. We have chosen to study the behaviour of cells from normal rat brains and from a glioma induced by ethylnitrosourea (ENU). A culture derived during the latent period of ENU-induced brain tumours has also been examined. These cultures have been characterized with respect to tumour formation and other features commonly associated with malignant transformation: growth in soft agar, fibrinolytic activity and surface topography (Roscoe & Claisse,
C. Tickle, A. Crawley and J. P. Roscoe

Table 1. Properties of the cultures

<table>
<thead>
<tr>
<th>Culture</th>
<th>Tumorigenicity in syngeneic rats</th>
<th>Growth in agar</th>
<th>Fibrinolytic activity (3)</th>
<th>Incidence of surface structures</th>
<th>Cell-cell and cell-substratum contact</th>
</tr>
</thead>
<tbody>
<tr>
<td>A15A5</td>
<td>+ (1)</td>
<td>+ (1)</td>
<td>+</td>
<td>Very high</td>
<td>Very poor</td>
</tr>
<tr>
<td>38D</td>
<td>+(2)</td>
<td>+(2)</td>
<td>+</td>
<td>Very high</td>
<td>Very poor</td>
</tr>
<tr>
<td>ARBOC9</td>
<td>-(3, 5)</td>
<td>-(3, 5)</td>
<td>-</td>
<td>Low</td>
<td>Good</td>
</tr>
<tr>
<td>ARBOC11</td>
<td>-(3, 5)</td>
<td>-(3, 5)</td>
<td>-</td>
<td>Very low</td>
<td>Very good</td>
</tr>
</tbody>
</table>

+, positive; -, negative.


We have investigated whether survival in the limb is correlated with malignant transformation and whether the cells invaded. Recently it has been suggested that the growth of cells sprinkled onto embryonic chick skin in organ culture may be correlated with malignancy (Noguchi, Johnson, O'Donnell & Petricciani, 1978). To try and obtain some idea of why some types of cells survive, whereas others do not, we have followed the fate of the cells implanted in the wing, at various times after grafting.

METHODS

Cell cultures

The control cells from normal brain tissue were ARBOC9 and ARBOC11. These are 2 clones from ARBO, a culture derived from the periventricular region of normal adult rat brain (Skidmore & Roscoe, unpublished results) and appear to have some characteristics of glia (see results). The tumour cells were glioma cells, A15A5. This is a clone of A15, a culture initiated from a cerebral glioma which arose in a rat following treatment of its mother during pregnancy with ENU (Lantos et al. 1976). If a single dose of ENU is administered to rats during the 3rd trimester of pregnancy, almost all the offspring develop tumours of the nervous system, of which a large proportion are brain tumours (Wechsler et al. 1969). The average latent period (dose 40 mg/kg) for cerebral tumours is 246 d. However, changes associated with malignant transformation can be detected in cells cultured from brains after around 111-112 d and before tumours are overt. We have investigated the behaviour of cells established from such a culture, 38D, which was prepared from a cerebrum of a rat 112 d after transplacental exposure to ENU (Roscoe & Claisse, 1978).

The cells have been tested for tumorigenicity and for some in vitro features often associated with malignancy. The results have been described elsewhere and are summarized in Table 1. A15A5 and 38D cells formed tumours when transplanted to syngeneic rats and possessed several features often correlated with malignant transformation, whereas ARBOC9 and ARBOC11 did not form tumours and did not display these characteristics.

The cells were maintained in Dulbecco's modification of Eagle's medium as described previously (Roscoe & Claisse, 1976, 1978) and were used in experiments when just sub-confluent. The cells were removed from the flasks in which they were growing following a standard treatment with 0.1 % trypsin + 0.001 M EDTA. Pellets of the brain cells or tumour cells, and of mixtures of these cells with embryonic quail wing cells, were prepared as described previously.
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(Tickle et al. 1978a). Quail cells were added to the cultured cells in some cases because this helped to bind the cells together in the pellet and made manipulation easier. Pieces of the pellets were cut out and grafted into holes cut in the dorsal surface of the wing buds of chick embryos (stage 20/21; Hamilton-Hamburger stages) in ovo (Tickle et al. 1978a). The embryos with the grafts were left to develop for various lengths of time following the operation, ranging from 5 h to 2 d.

The wings with the grafts were then fixed in half strength Karnovsky's fixative (Karnovsky, 1965) and the tissue was dehydrated and embedded in Araldite. Some grafts were prepared for electron microscopy and in these cases the tissue was rinsed after fixation in 0.1 M cacodylate buffer and then postfixied for 1 h at 4 °C in 1 % osmium tetroxide; thereafter the tissue was treated as described above. Sections, of thickness 1–2 μm, were cut serially and stained with 1 % toluidine blue. In cases where the tissue, prepared for light microscopy, contained quail cells, some of the sections were treated by a modified Feulgen technique so that the quail cells could be distinguished from the chick cells (Tickle et al. 1978a). For electron-microscopy, the grafts were found by taking off sections 1–2 μm thick and then ultra thin sections were cut. The thin sections were stained with lead citrate (Reynolds, 1963) and examined in a Philips EM 300 microscope.

RESULTS

Grafts of A15A5 glioma cells after 1 and 2 days

Ten grafts were made. In 2 cases we did not find the grafts in the wing and think that they had fallen out. In all the other 8 grafts many glioma cells were found. The cells appeared to be healthy and many mitoses were seen. We found extensive invasion of the chick mesenchyme by the glioma cells from the grafts (Fig. 1) in every case. Some individual cells were up to 440 μm away from the graft. However, the majority of cells were not so far away and also many remained at the site of the graft. The overall appearance of the limb was of a sprinkling of glioma cells within, and around, the graft region.

There did not appear to be any particular pattern of invasion. Glioma cells were not associated with ingrowing nerves as were sarcoma 180 and neuroblastoma cells (Tickle et al. 1978a). Also, they did not show the patterns of invasion that were typical of fibroblasts of the cell lines BHK, PyBHK, Nil 8 and HSV Nil 8 (Tickle et al. 1978a): glioma cells were rarely elongated along the ectoderm/mesenchyme border and showed no preference for this region, and were not particularly associated with blood vessels. In 2 grafts, a few glioma cells were found in the chick ectoderm. In 1 case (Fig. 2), the glioma cell appeared to be flattened along the ectoderm side of the basement membrane. We have found that positioning in the ectoderm is characteristic of cells from epithelial tumours (Tickle et al. 1978a, b) and that other cell types are rarely found in the ectoderm. Occasionally, there appeared to be some damage to the mesenchyme in the vicinity of the glioma cells (Fig. 1).

Grafts of 38D cells after 1 and 2 d

Seven grafts were made and in 2 cases, the graft was not found. The 5 grafts that we found were very extensive and cells were found in mitosis (Figs. 3, 4). Cells invaded the mesenchyme in all directions from the graft, sometimes in the form of elongated strands (Fig. 3). Invading cells were found in developing muscle or in loose connective tissue and showed no distinct patterns of invasion. The behaviour of the cells was
Fig. 1. A15A5 glioma cells (darkly staining) invading wing mesenchyme. Some of the glioma cells (arrowed) are at the border of ectoderm (e) with mesenchyme (m). Note also signs of erosion near glioma cells at *. Graft left 24 h.

Fig. 2. A15A5 glioma cell in ectoderm (e).

Fig. 3. Large graft (g) of 38D glioma cells showing invasion of mesenchyme by cells (arrowed) after 24 h. Note cell in graft in mitosis (double arrowed).

Fig. 4. 38D glioma cells (with darkly stained nuclei), invading mesenchyme. Note glioma cell in mitosis (arrowed). Graft left 48 h.
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Very similar to that of the A15A5 glioma cells: only occasionally were cells found along the mesenchyme/ectoderm border and only 1 cell was found in the ectoderm. In some cases there appeared to be destruction of host cells next to the implanted cells, but elsewhere there was close contact between graft and host cells with no sign of damage (Fig. 4).

Grafts of ARBOC9 and ARBOC11 cells after 1 and 2 d

Out of 28 grafts of normal brain cells left for 1 or 2 d, only 10 wings were found to contain any brain cells at all. In some cases the grafts may have fallen out but this could not account for all these cases. Also we know, in 4 cases, that the grafts had remained in the wing because the quail cells used to bind the brain cells together were found even though no brain cells were present. In the cases where brain cells were found, the graft consisted of a few brain cells within a small region of the
mesenchyme. This same result was obtained irrespective of whether the cells were ARBOC9 or ARBOC11 (Figs. 5, 6). Some of the cells within the graft looked unhealthy, with unusual nuclear morphology, but others appeared healthy (Fig. 6). The largest number of cells found in a wing 48 h after grafting was 23. In most cases less than 20 brain cells were found. In one case only 3 brain cells were present in a graft, which was made from a mixture with quail cells and had been left in the wing for 2 d. One of these cells (Fig. 7) was very large and possibly a giant cell. In other grafts left for 2 d the cells did not appear to be abnormally large (Fig. 8).

The brain cells in the grafts were found in a discrete region of the limb but were not closely apposed to each other (Figs. 5, 6). Some brain cells were separated from each other by chick cells. It is not clear whether this distribution is the result of invasion by the few brain cells remaining in the graft or infiltration of the graft by surrounding mesenchyme cells. It is, therefore, difficult, from these data, to draw any conclusions about the invasiveness of these cells.

**Time course of survival**

We examined grafts of ARBOC9 cells at various times after grafting, from 5.5 to 19 h, in order to trace what had happened to the brain cells. In grafts 5.5 h after the operation many brain cells were present and they looked healthy (Figs. 9, 10). The edges of the grafts were normally smooth, although occasionally cells from the graft appeared to bulge into the adjacent mesenchyme. We also found a few cells that had moved a short distance into the mesenchyme (Fig. 11) in these early grafts. We also saw a few mitotic figures in 1 graft fixed after 6 h. There were blood cells present within the grafts (Fig. 11). These were primary erythrocytes in mid- and late polychromatic stages (Lucas & Jamroz, 1961). Macrophages with phagocytic vacuoles containing debris, were found near the grafts.

The first signs of degeneration of brain cells appeared around 7 h after grafting and macrophages were found near the graft. A particular feature of the degeneration was the presence of circular holes containing debris (Figs. 12, 13). However, other cells appeared normal and were elongated, penetrating the adjacent mesenchyme. In grafts left 10 h many macrophages were present within the graft itself. There was

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Fig. 9. A graft of ARBOC9 brain cells 5.5 h after grafting.
Fig. 10. High power of Fig. 9 showing ARBOC9 cells in a graft which had been left for 5.5 h. Cells within graft look normal and there are no signs of cell death.
Fig. 11. Edge of a graft of ARBOC9 brain cells 6 h after the operation. One brain cell (arrowed) appears to be invading the mesenchyme adjacent to the graft. Also note blood cell (double arrowed) within graft.
Fig. 12. Graft of ARBOC9 cells after 10 h. Note invasion by brain cells (arrowed). Also cells in graft (g) showing degeneration.
Fig. 13. Graft region showing degeneration of ARBOC9 cells after 18 h in the limb. Note brain cell with distinctive scalloped outline (arrowed).
Fig. 14. Another graft showing degeneration of ARBOC9 cells 19 h after grafting. A few ARBOC9 cells are still present (arrowed) Note also several macrophages (double arrowed).
debris (Fig. 12) and there were also the clear spaces just described. After 18 h there was extensive degeneration and debris within the graft (Figs. 13, 14). Many macrophages were present. There were also signs of tissue erosion and large spaces between the remaining cells. Some of these brain cells have a distinctive scalloped outline. We have noticed that mesenchyme cells of the limb treated with high doses of
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X-irradiation sometimes have a similar morphology (Wolpert, Tickle & Sampford, 1979).

In contrast, grafts of A15A5 glioma cells appeared to flourish. Cells within a graft, fixed after 6 h, looked healthy and many dividing cells were seen (Fig. 15). Some of the cells from the graft had started to invade the adjacent mesenchyme (Fig. 15). There were blood cells within the graft but macrophages were not present. After 18 h, the A15A5 glioma cells formed a well-defined graft and there was extensive invasion of surrounding tissue (Fig. 16).

Morphology of implanted cells

The morphology of ARBOC9 cells in grafts left for short periods of time was examined with the electron microscope. It is of some interest to identify the origin of the cells in this brain clone. Many of the nuclei were very indented, with evenly distributed chromatin, apart from slight clumping next to the nuclear membrane (Fig. 17): these features have been described as being characteristic of astrocytes (Peters, Palay & Webster, 1976). Although the cytoplasm had a gross ‘matt’ appearance, it was difficult to resolve filaments at high magnification. Glial filaments have been found in these cells, using pellets of cells, which were fixed immediately after removal from tissue-culture dishes (P. Lantos, personal communication). The brain cells that invaded were elongated, occasionally with tapering processes.

A15A5 glioma cells often appeared to be rounded (Fig. 1). A characteristic shape for glioma cells was a triangle (Fig. 16). There were also some spindle-shaped cells. A few cells had elongated processes (20–25 μm long) at one end (Fig. 18). The nuclei of the cells showed ultrastructural features typical of astrocytes, but were not so frequently as irregular in outline as those of ARBOC9 cells. Again, it was difficult to find cytoplasmic fibrils but they have been observed under other conditions (Lantos et al. 1976). 38D cells had a similar appearance to A15A5 cells in the limb. In particular, we again noticed cells with triangular outlines. Their general ultrastructural appearance (Fig. 19) is again consistent with an origin from astrocytes. Both cultures formed tumours histologically identifiable as glial (Lantos et al. 1976; Claisse, Lantos & Roscoe, 1978).

DISCUSSION

Comparison of the behaviour of normal and malignant cells

We have found that cells from normal rat brain tissue do not survive when implanted in the embryonic chick wing bud. In contrast, cells from a glioma and from a carcinogen-treated rat brain survive well. This is in accord with our previous findings that normal BHK and Nil 8 hamster cells did not survive as well as their transformed derivatives in the chick wing (Tickle et al. 1978a). However, the difference in survival between cells from normal rat brain and tumorigenic brain cultures was much more marked than that found between normal and transformed fibroblasts. In addition, we have preliminary data that MRC5 cells, a normal human diploid cell line (Jacobs,
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Jones & Baille, 1970), similarly do not survive well and few cells can be found 1 day after grafting.

In a similar vein, recent findings on a number of different tumour and normal cell lines (Noguchi et al. 1978) suggest that there is a marked difference between normal and malignant cells in their ability to grow on embryonic chick skin in organ culture, and in some cases the normal cells were necrotic. In addition, there may be similar differences between normal and transformed 3T3 cells in their survival on the chick chorio-allantoic membrane (CAM) (Scher, Haudenschild & Klagsbrun, 1976).

We should point out, however, that not all the normal cells implanted in the limb degenerated and disappeared. This small number of surviving cells may be variants and, indeed, a minority of normal cells from ARBOC9 do produce plasminogen activator as measured by fibrinolytic activity (Hince & Roscoe, 1978). Tumour-forming variants can also be detected if large numbers of normal cells, such as BHK cells, are injected into animals (Jarrett & Macpherson, 1968).

We also compared the invasiveness of these normal and tumorigenic cells in the limb. After 2 d, so few normal cells survived that no conclusions could be drawn. However, by looking soon after grafting, we found that the normal brain cells penetrated short distances into the mesenchyme of the wing. Preliminary data suggest that MRC5 cells also behave in this way. In addition, invasive behaviour was exhibited by cells in grafts of BHK and Nil 8 (Tickle et al. 1978a) and when BHK cells were placed onto the CAM in culture, they infiltrated slowly (Easty & Easty, 1974). In contrast, Scher et al. (1976) could not find any signs that normal 3T3 cells penetrated the CAM. However, other recent experiments suggest that normal fibroblasts from cell lines such as WI-38 (Noguchi et al. 1978) invade embryonic chick skin. It seems likely, therefore, that the ability to invade is not a characteristic that distinguishes malignant from normal cells. Cells from ‘normal’ cell lines can be invasive. There are, in addition, many other instances of invasion in vivo by normal cells, such as trophoblast and polymorphonuclear leukocytes, as well as those involved in normal morphogenesis.

Various tumour cells including neuroblastoma cells and cells from breast tumours survive when implanted in the chick wing (Tickle et al. 1978a, b). In addition, normal embryonic tissues from various sources, such as quail liver and mesonephros survive and divide (Tickle, Goodman & Wolpert, 1978). Whatever the necessary characteristics enabling survival are, they appear to be shared by both malignant and embryonic tissue and it has often been suggested that tumour and embryonic cells have common properties. All these data support the idea that survival and growth of cells in embryonic tissues may be a characteristic of tumour cells and a more useful indicator of malignancy than invasiveness.

Fig. 17. ARBOC9 brain cells within a graft left 6 h in the limb. Note indented nuclei with chromatin distribution typical of astrocytes.

Fig. 18. A15A5 glioma cells (g) in chick mesenchyme; 1 glioma cell has a very long process. Also note swollen cisternae of endoplasmic reticulum (arrowed) in another glioma cell.

Fig. 19. 38D cell showing nuclear morphology typical of an astrocyte. Note lipid droplet (l).
Patterns of invasion of malignant brain cells

The cells from the glioma and the carcinogen-treated rat brain behaved in the same way. The patterns of invasion were not the same as those observed in grafts of fibroblast cell lines, BHK, PyBHK, Nil 8 and HSV Nil 8 (Tickle et al. 1978a, c). This is consistent with the morphology of the glioma and brain cells, which suggests that these cells are glia rather than fibroblasts. The occasional positioning of cells in the limb ectoderm suggests that cells of these gliomas behave in a way intermediate between that of cells from fibroblastic and epithelial tumours.

Mechanisms involved in cell survival

It would be important to find out the factors that determine whether cells survive, and the basis for the difference between the normal and tumour cells. Initially, we checked whether this difference merely arose from some trivial cause, such as damage to the cells during preparation for grafting. We could find no indication that the handling of the cells could explain the results: the colony-forming ability was about the same for both normal and tumour cells. In addition, we counted the numbers of normal and tumour cells in our grafts. Although there were about 12% fewer cells in the normal grafts than in the tumour grafts this could not explain the difference in the numbers of cells in these kinds of grafts after 1 d.

It is possible that a nutrient required for the maintenance of normal cells and their growth is absent from the limb. Another possibility is that the limb environment is toxic to the normal cells. For instance, if the limb were anoxic this might explain the results, as malignant cells are more resistant to lack of oxygen than normal cells. There seems little reason to suppose that the limb is anoxic, although the remarkable insensitivity of the limb bud cells to high doses of X-irradiation (Wolpert et al. 1978) would fit this.

As far as we know, the mechanisms involved are not understood in any of the circumstances where there is a difference in survival and growth between normal and malignant cells, e.g. in syngeneic animals, nude mice and growth in soft agar. There appears to be a correlation both between growth in soft agar and tumour production in nude mice (Freedman & Shin, 1974), and between growth in nude mice and in chick skin (Noguchi et al. 1978). There is also a good correlation between tumour formation in syngeneic animals and growth in agar in these brain cells (Roscoe & Claissie, 1976, 1978; Lantos et al. 1976). In the 4 cases tested so far, survival in the chick limb bud correlates with these 2 parameters.

It is tempting to suggest that the lack of growth and the absence of tumours when normal cells are implanted into syngeneic animals or nude mice or embryonic tissues may be reflexions of the same mechanisms which result in the inability of normal cells to grow in soft agar. The anchorage dependence of cell division has been used as a criterion for normality of cells (Stoker, O'Neill, Berryman & Waxman, 1968), transformation often resulting in lack of anchorage dependence for growth. However, if normal cells are provided with a solid substratum they will form colonies in agar (Stoker et al. 1968) or tumours in mice (Boone, 1975; Boone, Takeichi, Paranjpe &
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Gilden, 1976). One interpretation would be that this procedure in some way allows selection of 'spontaneously' preneoplastic cells, possibly by permitting cells to divide soon after implantation. Failure to form tumours in animals suggests that normal cells cannot use the surfaces of other normal cells, for example, as substrata for growth. Few data are available on this point, but 3T3 cells do not divide in aggregates for the first 3 d following formation (Carrino & Gershman, 1977). Another mechanism which may result in normal cells not forming tumours, is the absence of vascularization of the graft (Giovanella, Stehlin & Williams, 1974) because normal cells do not produce angiogenesis factor (Folkman, 1974).

We should point out that in our experiments any conventional immune response is ruled out, as this has not developed in chick embryos at this stage. However, macrophages were associated with the degenerating grafts. It has been suggested that the ability of melanoma cells to form tumours is related to the production of plasminogen activator, which in some way prevents infiltration of macrophages and other possible 'killer' cells into inocula (Newcomb, Silverstein & Silagi, 1978). Of the cells used in this work, A15A5 and 3BD which survive well, have high fibrinolytic activity, while ARBOC9 and ARBOCi 1 do not. Our results show that macrophages ingest and clear away dying cells. Their appearance seems to coincide with, rather than precede, signs of cell degeneration in the graft, but we cannot eliminate the possibility of a more active role.

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