Interactions between normal mammary epithelial cells and mammary tumour cells in a model system

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Abstract. Normal mammary epithelial (NME) cells and MCF-7 cells aggregate and grow as spheroids when cultured on extracellular matrix derived from Engelbreth/Holmes/Swarth (EHS) tumour. NME cells stop dividing and differentiate but MCF-7 cells continue to proliferate, although growth is counterbalanced by cell death. In mixed cultures of NME cells and MCF-7 cells, the two cell types form mixed aggregates but then segregate to form well separated domains, often joined by only a narrow neck of cells. In these mixed cultures the growth of MCF-7 cells is inhibited by a factor secreted by NME cells into the medium.

INTRODUCTION

Interactions between tumour cells and surrounding normal cells are likely to be important in the ontogeny of tumours. In tissue culture such interactions have been found to suppress the growth of transformed cells (Stoker, Shearer & O'Neill 1966, Stoker 1967) by a mechanism that may involve cell–cell communication through gap junctions (Mehta, Bertram & Loewenstein 1986, Bignami *et al.* 1988, Dotto, Weinberg & Ariza 1988, Martin *et al.* 1991), secretion of juxtacrine factors or adhesion signalling (Rosenburg & Massague 1993, Gradi *et al.* 1995). Whether the growth of tumour cells is also suppressed in tissues by the same interactions is not known.

A major difference between tissues and tissue culture is the presence of extracellular matrix (ECM) in the former. It is becoming widely apparent that ECM effects the behaviour of cells (Gimond & Aumailley 1992, Streuli *et al.* 1993, Lin *et al.* 1995) and may well affect cell–cell interactions.

One well-studied example of the influence of ECM on cell behaviour is the formation of mammospheres by normal mammary epithelial (NME) cells taken from pregnant mice. NME cells in monolayer culture continue to divide until confluent, but in the presence of Engelbreth/Holmes/Swarth (EHS) matrix, they stop dividing and aggregate to form spheres (Streuli *et al.* 1993, Barcellos-hof *et al.* 1989, Lin *et al.* 1995). Mammospheres become hollow and milk is secreted vectorially into the lumen-like space. The mammospheres thus recapitulate many of the features of the alveoli of mammary glands. This behaviour is not confined to primary cells as immortal mammary epithelial cell lines such as CID9, HMT-3522

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and MCF10A, likewise form mammospheres (Petersen *et al.* 1992, Boudreau, Werb & Bissell 1996, Weaver *et al.* 1997). In this latter case, the presence of $\beta 1$ integrin, which binds to a variety of ECM components, has been shown to be central to mammosphere formation (Weaver *et al.* 1997). Tumour cells such as MCF-7 cells also form mammosphere-like structures on EHS matrix but unlike normal cells, these cells continue to grow (Peterson *et al.* 1992).

The difference in growth between NME cells and MCF-7 cells in the presence of ECM provides a system for analysing mechanisms of growth suppression through cell-cell interactions. The present study investigated the behaviour of these two cell types on EHS matrix in mixed and separate culture. NME cells were found to interact more strongly with the matrix, cells in mixed aggregates segregated almost completely into separate NME and MCF-7 domains and normal cells secreted a soluble factor that suppressed the growth of the tumour cells.

MATERIALS AND METHODS

Cell culture

Primary NME cells were isolated from 14-day time-mated pregnant CD-1 mice (Charles River Laboratories, Margate, Kent, UK) by collagenase IV (Lorne Laboratories, Twyford, UK) digestion of dissected mammary tissues for 90 min at 37°C with continual rotary shaking in a 1 : 1 mixture of Hams F12 and Medium 199 tissue culture media containing 0.2% sodium bicarbonate, 0.5 mM HEPES, 1 mM glucose, 5 μ g insulin/ml, 1 μ g prolactin/ml, 100 μ g cortisol/ml. Tissue debris was removed by filtration through 50 μ m pore nylon mesh (Lockerter, Warrington, UK) and cells pelleted by centrifugation (10 min, 600 *g*, at room temperature) and washed twice with fresh medium. The cells were seeded in the above medium containing 10% fetal calf serum which was changed daily with fresh serum-free medium. All cell lines were cultured in the same culture media containing 10% fetal calf serum.

Cells were seeded into 35 mm and 50 mm dishes either directly onto the tissue culture plastic, or on top of a layer of EHS extracellular matrix (Becton-Dickinson, Oxford, UK), spread at 0.26 μ l/mm² and allowed to set. In co-culture experiments or experiments comparing the behavioural properties of cells (e.g. [³H]thymidine incorporation), the cells were cultured in serum-free medium supplemented with insulin, prolactin and cortisol at the above concentrations.

Histology

Cells were released from EHS matrix using dispase (Becton-Dickinson) and allowed to settle by gravity before resuspension in a small volume of culture media. The cells were pipetted onto OTC freezing compound on a cryostat chuck, and frozen at -80°C. Alternatively cultures were fixed in freshly made 4% paraformaldehyde and the appropriate area of the dish was cut using a scalpel. The fixed materials were lifted from the cut region of the dish and embedded in LR-White resin prior to sectioning.

Measurement of incorporation of [³H]thymidine

[³H]Thymidine (0.185 MBq; Amersham, Little Chalfont, UK) in 5 ml medium was added to each 35 mm dish of cells for the final 16 h of culture. The medium was removed and the cultures rinsed with phosphate-buffered saline (PBS). The cells were scraped into 100 μ l 1%

SDS and precipitated by the addition of 10 ml 10% (w/v) trichloroacetic acid (TCA) at 4°C for 30 min. The mixture was poured through a glass fibre class A filter (Whatman), rinsed twice in 10 ml 5% trichloroacetic acid and once with 10 ml ethanol. After drying, the filter was placed in 5 ml scintillation fluid and radioactivity counted on a 1600 TR liquid scintillation counter (Canberra Packard, Berkshire, UK).

Transfection with β -galactosidase and staining

MCF-7 cells were co-transfected with the plasmids pSV1 (β -galactosidase expression vector) and pNeo (plasmid containing the G418 resistance gene) using GIBCO Lipofectamine™ (Paisley, UK) according to manufacturer's instructions. G418-resistant clones were selected (500 μ g/ml G418) and stained for β -galactosidase activity. Cultures were fixed in 1% formaldehyde and 0.1% glutaraldehyde in PBS and then stained for 2–3 h with freshly prepared stain in PBS containing 1 mg/ml X-galactosidase, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 2 mM magnesium chloride.

RESULTS

Behaviour of NME and MCF-7 cells on EHS matrix

Previous studies (e.g. Barcellos-hof *et al.* 1989) have shown that normal mammary epithelial cells (NME cells) taken from pregnant mice and MCF-7 cells form spheroid structures on extracellular matrix isolated from EHS tumours. Whereas cells in the former cultures stop growing and differentiate, the latter cells continue to grow. These two cell types were therefore chosen to investigate interactions between normal and transformed cells, in particular any growth suppressive activity by normal cells on transformed cells in EHS matrix.

To allow for the different growth rates (Peterson *et al.* 1992 and the authors' observations), NME cells were seeded on EHS matrix at 10 times the density of MCF-7 cells (3×10^6 NME cells/cm² and 3×10^5 MCF-7 cells/cm²). Growth was measured by the incorporation of [³H]thymidine and was compared to parallel cultures seeded at the same densities on plastic (Figure 1). [³H]Thymidine incorporation by both NME and MCF-7 cells on EHS matrix was reduced (up to 95% at 2 days for NME cells and 80% at 2–5 days for MCF-7). Over 8 days, incorporation by NME cells on EHS matrix dropped steadily while incorporation by MCF-7 cells remained approximately constant with no measurable increase in spheroid diameter indicating that the rate of proliferation was matched by the rate of cell loss. Analysis of NME and MCF-7 cell spheroids by dUTP/transferase showed the presence of degraded DNA (data not shown) indicating cell death.

Within 24 h, both types of cells aggregated on EHS matrix into rather evenly distributed spheroids. By day 7, spheroids of NME cells became hollow with a mostly single layer of cells surrounding a central lumen-like space (Figure 2a), whereas MCF-7 spheroids were mostly filled with cells with few, irregularly distributed small spaces (not shown). After 9 days the spheroids of NME cells started to degenerate but the spheroids of MCF-7 cells survived for up to 30 days (Figure 2b). Increasing the seeding density 10-fold for either cell type resulted in tubular structures rather than spheroids, as found by others (Berdichevsky *et al.* 1994).

Spheroids did not form when the two cell types are cultured on laminin, fibronectin, collagen type 1, or collagen type 4 alone. Both cell types require the presence of a number of ECM components but interact differently with EHS matrix. Matrix around NME spheroids, but not MCF-7, appeared to be pulled off the dish to leave a clear halo. If inert clay beads were

Figure 1. Incorporation of [^3H]thymidine by cultures of NME cells and MCF-7 cells cultured on plastic (monolayer) and on EHS matrix over 8 days. NME cells or MCF-7 cells were seeded at 3×10^6 cells/cm 2 or 3×10^5 cells/cm 2 , respectively, on plastic or on EHS matrix and cultured for up to 8 days. Media was changed daily and [^3H]thymidine added for the final 16 h at each time point before harvesting and measuring the incorporation of radioactivity into trichloroacetic acid-insoluble material (see *Materials and methods*). Three replicate cultures were used for each data point.

sprinkled on the EHS matrix while it was setting, they later moved towards the NME spheroids as the matrix was drawn over the structures whereas formation of MCF-7 spheroids did not disturb the surrounding matrix and hence the distribution of the clay beads (Figure 3).

Interactions of NME and MCF 7 cells in spheroids in co-culture on EHS matrix

To examine interactions between NME and MCF-7 cells in mixed culture on EHS matrix, MCF-7 cells were labelled with β -galactosidase. Cells were cultured on EHS matrix at the same seeding densities (3×10^6 NME cells/cm 2 and 3×10^5 MCF-7 cells/cm 2), to give a starting ratio of 10 NME cells to 1 MCF-7 cell. The co-cultures produced double-lobed structures (Figure 4b). These structures were formed by cell aggregation into mixed structures of assorted shapes (Figure 4a) followed by segregation of NME and MCF-7 cells into separate domains (Figure 4b) by 3 days. Sections through the double-lobed structures confirmed the segregation of NME cells and MCF-7 cells (Figure 4c), although occasional isolated MCF-7 cells were found in the NME domains. Increasing the seeding density 10-fold (but retaining the ratio of 10 NME cells to 1 MCF-7 cell) gave rise to branching tubular structures by 3 days that consist of both cell types (Figure 4d). However, the two cell types segregate from one another to form distinct domains within the tubular structures.

Measuring rates of proliferation by [^3H]thymidine incorporation showed that NME cells

Figure 2. Spheroid structures of NME and MCF-7 cells cultured on EHS matrix. NME cells (a) and MCF-7 cells (b) were cultured on EHS matrix (see text for details). The sections shown in (a) and (b) are from 8 day and 21 day cultures. The white arrow in (b) shows the debris in the cell-free area. Bars: 25 μ m.

Figure 3. Distribution of clay beads in NME and MCF-7 cell cultures on EHS matrix. Clay beads were added to the setting EHS matrix before addition of NME cells (a,b) and MCF-7 cells (c,d) at low density on to EHS matrix. Micrographs were taken after 3 days of culture. The clay beads are drawn towards and over the NME cell spheroids, but are not disturbed by the MCF-7 cell spheroids. Bars: (a) and (c), 200 μ m; (b) and (d), 50 μ m.

inhibit the growth of MCF-7 cells (Figure 5). In the mixed cultures, incorporation attributable to MCF-7 cells was reduced at all time points and reached a maximum of 90% by 3 days. Although there was marked loss in growth, the MCF-7 cells continued to proliferate dominating the tubular structure by 8 days (data not shown)

Nature of the growth inhibition by NME cells

The lack of extensive intimate contact between NME and MCF-7 cells at 3 days, when the two cell types have segregated and growth inhibition is maximal, suggests that the inhibition

Figure 4. Spheroid and tubular structures of NME and MCF-7 cells co-cultured on EHS matrix. NME cells and MCF-7 cells were seeded onto EHS matrix at densities of 3×10^6 cells/cm² and 3×10^5 cells/cm², respectively, for low density (ac) or 10-times higher for high density (d) cocultures. The MCF-7 cells were first transfected with β -galactosidase. Cultures were fixed and stained with X-gal after 10 h of culture (a) or 3 days (bd) or 8 days of culture (inset in (b)). (a), (b) and (d) show phase contrast micrographs and (c) is from a transverse section. Bars: (a) 50 μ m; (b) 100 μ m; (b inset) 50 μ m; (c) 50 μ m; (d) 40 μ m.

Figure 5. Incorporation of [³H]thymidine by separate and mixed cultures of NME and MCF-7 cells on EHS matrix. Separate cultures and co-cultures (10 : 1) of NME and MCF-7 cells were established on EHS matrix at seeding densities of 3×10^6 cells/cm² for NME and 3×10^5 cells/cm² for MCF-7 cells. Media were changed daily and [³H]thymidine added for the final 16 h at each time point before harvesting and measuring the incorporation of radioactivity into trichloroacetic acid insoluble material (see *Materials and methods*). Three replicate cultures were used at each time point.

occurs by factors secreted into the medium. This was confirmed by transferring medium, conditioned by NME cells, to MCF-7 cells and measuring [³H]thymidine incorporation. Conditioned medium was prepared by seeding NME cells on plastic or on EHS matrix. Media was conditioned on these cultures for 1 day between days 4 and 5 of culture, or for 2 days between days 3 and 5 of culture. Conditioned medium was transferred onto subconfluent monolayer cultures of MCF-7 cells which were then grown for 1 day before

Figure 6. Effect of NME cell-conditioned media on the incorporation of [^3H]thymidine by MCF-7 cells. Conditioned medium was prepared by seeding NME cells at a density of 3×10^6 cells/cm 2 on plastic (monolayer) or on EHS matrix. Media was conditioned on these cultures for 1 day between days 4 and 5 of culture, or for 2 days between days 3 and 5 of culture. The conditioned medium was placed on 5-day cultures of MCF-7 cells seeded at a density 3×10^5 cells/cm 2 on plastic dishes. One day later [^3H]thymidine was added for 16 h before harvesting and measuring the incorporation of radioactivity into trichloroacetic acid-insoluble material (see *Materials and methods*). The unconditioned medium was incubated for 1 or 2 days as appropriate at 37°C. Three replicate cultures were used for each data point.

measuring [^3H]thymidine incorporation as described above. The incorporation was compared to parallel cultures of MCF-7 cells treated with fresh medium that had been incubated at 37°C for 1 or 2 days.

In the presence of medium conditioned for 1 day by NME cells in monolayer culture or on EHS matrix, the [^3H]thymidine incorporation was partially reduced but 2-day conditioned medium resulted in a near complete inhibition of incorporation (Figure 6). Medium conditioned by NME cells on EHS matrix was more potent. The inhibition was not a result of the depletion of nutrients or factors in the medium as shown by adding glucose, hormones, fetal calf serum or 50% fresh medium to the conditioned medium. In all cases there was still a marked inhibition of [^3H]thymidine incorporation (Figure 7). In addition, fresh medium conditioned by MCF-7 cells did not inhibit incorporation. These data suggest NME cells secrete an inhibitory factor.

Such an inhibitor has already been described (Mali *et al.* 1994). NMuMG cells, a murine cell line established from normal mammary epithelial cells, secrete the ectodomain of the

proteoglycan syndecan 1, which is able to inhibit the growth of MCF-7 cells. Comparative experiments showed that 2-day conditioned media prepared from NME cells and from NMuMG cells both reduce, to similar extents, [^3H]thymidine incorporation in MCF-7 cells (Figure 7) and, as found by Mali *et al.* (1994), the inhibitory factor is heat stable (Figure 7).

DISCUSSION

Behaviour of NME and MCF-7 cells in separate culture

Whilst both NME cells and MCF-7 cells form spheroids on EHS matrix there are two important differences. Firstly, MCF-7 cells continue to grow, albeit at a lower rate than on plastic, whilst the NME cells stop growing and differentiate. Secondly, there appears to be more limited interaction between MCF-7 cells and EHS matrix than between NME cells and matrix.

The results of Peterson *et al.* (1992) showed continued growth of MCF-7 cells on EHS matrix but this was measured only by examining the [^3H]thymidine labelling index. The present results also show MCF-7 cells continue to grow on EHS matrix but the rate is considerably less than in parallel cultures growing on plastic. This loss of growth is accompanied by cell death in the spheroids. The cause of cell death is not clear but it leads to the accumulation of debris in the spheroids.

Unlike the spheroids of NME cells, the spheroids of MCF-7 cells do not draw in surrounding matrix suggesting there is less interaction between MCF-7 cells and EHS matrix components. Other transformed cells of mammary epithelial origin do not form spheroids on extracellular matrix (Peterson *et al.* 1992, Hirschi *et al.* 1996) suggesting that even with the limited interaction, MCF-7 cells are responding either to the presence of matrix or the absence of plastic.

It has been suggested that gap junction formation, resulting from expression of connexins, is necessary for the formation of spheroids by mammary epithelial cells (Hirschi *et al.* 1996). Connexins are a family of transmembrane proteins required for gap junction formation (see Goodenough, Goliger & Paul 1996 for review). It has not been possible to detect expression of connexin 43, the major connexin of mammary epithelial cells, in MCF-7 cells and there is no detectable gap junctional communication (measured by Lucifer Yellow microinjection) between MCF-7 cells growing on plastic or during spheroid formation on matrix (data not shown) showing that gap junction formation is not always required to form spheroids.

Behaviour of NME and MCF-7 cells in mixed culture

In mixed cultures, the results show direct and indirect interactions between MCF-7 cells and NME cells on EHS matrix. Initially the two cell types form composite structures but over time they segregate to form separate domains. The spheroids usually remain attached, albeit through a thin neck of cells, and at high seeding densities the two cell types form tubular structures that contain both cell types. This shows there is restricted interaction between NME and MCF-7 cells while there are preferential homologous interactions that give rise to segregation. The preferred homologous interactions might be a result of the two cell types originating from different species (mouse and human) although segregation in monolayers in some instances is based upon cell type and not species of origin (Prowse, Cadwallader & Pitts 1997).

The indirect interactions result in a marked loss of growth of MCF-7 cells. NME cells secrete an inhibitory factor that reduces the growth rate of MCF-7 cells, as measured by

- 1 - Fresh medium
- 2 - Fresh medium - heat treated
- 3 - Medium conditioned by NME cells
- 4 - Medium conditioned by NME cells - heat treated
- 5 - Medium conditioned by NME cells + fresh medium (1:1)
- 6 - Medium conditioned by NME cells - freeze thawed
- 7 - Medium conditioned by NME cells + glucose
- 8 - Medium conditioned by NME cells + hormones
- 9 - Medium conditioned by NME cells + FCS
- 10 - Medium conditioned by NMuMG cells
- 11 - Medium conditioned by NMuMG cells - heat treated
- 12 - Medium conditioned by NMuMG cells + fresh medium (1:1)
- 13 - Medium conditioned by MCF-7 cells
- 14 - Medium conditioned by MCF-7 cells - heat treated

Figure 7. Effects of treating or supplementing conditioned media from NME, NMuMG and MCF-7 cells. Two-day conditioned media were prepared as described in the legend to Figure 6 from cultures of NME, NMuMG, or MCF-7 cells. The effect of the conditioned medium on the incorporation of [3 H]thymidine by cultures of MCF-7 cells was measured after the indicated treatments which included: heating to 65°C for 10 min (heat-treated); freezing at 20°C for 10 min before thawing at room temperature; mixing 1:1 with fresh medium; or adding glucose (100 μ l, 10 mM), hormones (see *Materials and methods*), or 0.5 ml fetal calf serum to 5 ml conditioned medium.

thymidine incorporation, by as much as 90%. Similar inhibition has been reported with medium from a normal mammary epithelial cell line (NMuMG) and the active factor identified as the ectodomain of syndecan-1, a proteoglycan produced by many normal epithelial cells (see Rapraeger & Ott 1998 for review). As yet there is no report of primary cells secreting the ectodomain of syndecan-1 and further work is required to fully characterize the inhibitory factor from NME cells.

The type of inhibition observed in this model system is similar to that seen in monolayer culture involving paracrine interactions (Rosenburg & Massague 1993, Gradi *et al.* 1995). However, the extent of inhibition is more marked and this appears in part to be a result of the NME cells producing more inhibitory factor in EHS matrix than in monolayer culture. Experiments with other combinations of normal and transformed cells in EHS matrix may likewise increase growth suppression by the normal cells over that seen in monolayer culture. In addition, there is extensive inhibition of proliferation of MCF-7 cells by EHS matrix alone. The combined effect of both leads to a reduction of [³H]thymidine incorporation and hence growth of MCF-7 cells of > 95%. It seems likely therefore that MCF-7 cells, and possibly other transformed cells, may grow more slowly in solid tumours than in conventional monolayer culture.

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