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Lack of Correlation Between Transformed Characteristics in Culture and Tumorigenicity of Mouse Mammary Tumour Cells

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Abstract—*Androgen responsive and unresponsive Shionogi 115 mouse mammary carcinoma cells have been examined for anchorage-independent growth and tumorigenicity in nude mice. The two cell types exhibit transformed and normal growth characteristics respectively, but both give rise to tumours in nude mice. No correlation between tumorigenicity and transformed characteristics including anchorage-independent growth could be demonstrated.*

INTRODUCTION

THE INVESTIGATION of neoplastic transformation *in vivo* is complicated by the lack of defined phenotypic changes which distinguish between normal and tumour cells. Several cellular parameters which make this distinction have been identified *in vitro* using chemically and virally transformed cell lines, and attempts have been made to relate these parameters to tumorigenicity *in vivo*. Thus, alterations in cell morphology, reduced serum requirement, decreased density regulation, anchorage independence, increased proteolytic activity, changes in membrane structure and disorganisation of the cytoskeleton have all been implicated in the transformation process *in vitro* [1–3]. The correlation between these parameters and tumorigenicity *in vivo* is less well-defined, however, and exceptions exist for all of them. At the present time the transformed characteristics regarded as being most closely related to tumorigenicity are anchorage-independent growth and disorganisation of the cytoskeleton [4, 5].

We have been using a cell line derived from the androgen-responsive Shionogi 115 (S115) mouse mammary carcinoma to investigate the role of steroid hormones in regulating cell proliferation. S115 cells retain their androgen responsiveness for long periods of time when cultured in the presence of testosterone. When deprived of testosterone for 2–3 weeks, however, responsiveness is lost without a significant reduction in androgen receptor levels [6]. We have shown that the growth characteristics and morphology of androgen-responsive and unresponsive cells differ greatly [7]. The androgen-responsive cells show many of the characteristics of transformed cells in culture described above. The androgen-unresponsive cells, however, possess the characteristics of normal cells. In addition, androgen-responsive S115 cells grow well in suspension culture whereas the unresponsive cells are absolutely dependent on substrate attachment for growth [7], a finding previously noted by others [8]. Furthermore, the loss of anchorage independence following androgen deprivation is the parameter most closely related to loss of hormone responsiveness [9].

The S115 cells thus provide a system in which cells derived from the same original line can show either normal or transformed characteristics. It was felt that useful information about the relationship between transformation and tumorigenicity would be gained by investigating tumour formation after injection of both types of cells into nude mice.

MATERIALS AND METHODS

Cell culture

Stock cells were routinely cultured in a

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humidified atmosphere of 5% carbon dioxide in air at 37°C in Dulbeccos modified Eagle's medium (DMEM) supplemented with 2% foetal calf serum (Gibco BioCult, Glasgow, Scotland) and 40 nM *N* - 2 - hydroxyethylpiperazine - *N'* - 2 - ethane-sulphonic acid (Sigma Chemical Co.); 35 nM (0.01 μm/ml) testosterone (Steraloids Ltd., Croydon, England) was added where required. Cells were subcultured twice weekly. Stock lines of both androgen-responsive and androgen-unresponsive lines were held.

Cell growth assays

Cells from stock plates were suspended in 0.05% trypsin buffered with 0.02% EDTA (pH 7.3), counted on a haemocytometer and added to prepared growth medium with or without testosterone at the desired concentration. Aliquots (5 ml) of cell suspension were placed in 50 mm plastic tissue culture dishes for monolayer growth or 50 mm plastic Petri dishes for growth in suspension culture. At certain times the cells were harvested for counting. Monolayer cultures were suspended in trypsin and diluted with Isoton (Coulter Electronics Ltd., Harpenden, England), suspension cultures were transferred to 20-ml screw-topped plastic universal containers, centrifuged at 700 *g* for 5 min and the cell pellet dissociated with trypsin and diluted with Isoton. Cell numbers were estimated from triplicate Coulter Counter readings. All cell counts were expressed as the mean of triplicate plates; standard errors were always less than 5% of the mean.

Cell growth in agarose gels

Two-millilitre aliquots of 0.5% LGT agarose (Marine Colloids, Inc.) in DMEM were placed in 50-mm plastic Petri dishes and allowed to solidify at 4°C. The dishes were then warmed to 37°C before adding the cells. Trypsinised cell suspensions were added to DMEM containing 6% foetal calf serum with or without 10^{-7} M testosterone as required at a concentration of 7500 cells/ml. One volume of cell suspension was then mixed with two volumes of 0.5% agarose in DMEM and 2-ml aliquots were spread over the basal layers to give 5000 cells/plate. After 10 min at 37°C during which the cells settled to the interface of the two layers, the gels were allowed to solidify at 4°C for 5 min. A layer of 2 ml of normal growth medium was added to prevent drying of the gels and the plates were incubated at 37°C for 14 days. At the end of this time the gels were fixed for 30 min in formol saline, stained for 1 min in 1% polychrome methylene blue and destained overnight in distilled water. Cell colonies of minimum diameter 0.2 mm were counted across four diagonals on a Biotran II automatic colony counter (New Brunswick Scientific Co. Inc.) and the mean of four counts calculated. Results were expressed as the mean of triplicate plates, standard errors were always less than 10% of the mean.

Tumour growth in nude mice

Male athymic nude mice (nu/nu) were used at four months of age. When castrated animals were used, castration was carried out one month before injection of cells. S115 cells were suspended in sterile saline at a concentration of 10^7 cells/ml and 0.1 ml was injected subcutaneously into the flank of each mouse. Appearance of tumours was observed weekly for three months. After tumour development mice were kept until death or sacrifice. The length and width of each tumour was measured at intervals and the mean of length and width was used as an index of tumour size. Tumours were given a score of 1–4 according to size and the mean score per tumour was used as an index of tumour growth for each group of 8 mice. The tumour size at first detection was 1–2 mm in diameter.

Culture of tumour cells

Excised tumour tissue was chopped roughly with scissors and was then chopped into pieces of 1 mm^3 using a Mickle chopper (Mickle Engineering). The tissue was shaken in 0.25% trypsin for 15 min at 37°C and the large lumps allowed to settle out under unit gravity. The supernatant containing cell clumps was centrifuged and the pellet was suspended in growth medium containing 10% foetal calf serum and plated into tissue culture dishes. When a large proportion of the cells had attached to the dish after a few hours, the debris was removed and fresh medium supplied. After 24 hr the cells were changed to medium containing 2% foetal calf serum.

RESULTS

Anchorage dependence

Growth in plastic Petri dishes. The androgen-responsive cells proliferate almost as well in plastic Petri dishes to which they cannot attach as in a monolayer if testosterone is present (Fig. 1). In the absence of testosterone there is no growth in suspension. The androgen-unresponsive cells grow very poorly in suspension culture in the presence or absence of testosterone.

Growth in agarose gels. When grown in the presence of testosterone approximately 6% (6.05 ± 1.20%) of the androgen-responsive cells formed colonies in gels. Androgen-responsive cells in the temporary absence of testosterone and androgen-unresponsive cells in either the presence or absence of testosterone produced no colonies. The plating efficiency of both cell types in the presence or absence of testosterone is approximately 43% in plastic tissue culture dishes.

Tumour formation in nude mice

Both androgen-responsive and unresponsive cells formed tumours in intact and castrated mice after a period of at least six weeks (Fig. 2; Table 1). The rate of tumour growth by the androgen-unresponsive cells was the same in both intact and castrated animals, and in both types of animal was faster than that of the androgen-responsive cells. The latter showed androgen sensitivity *in vivo*, producing tumours much more rapidly in intact animals than in the castrates.

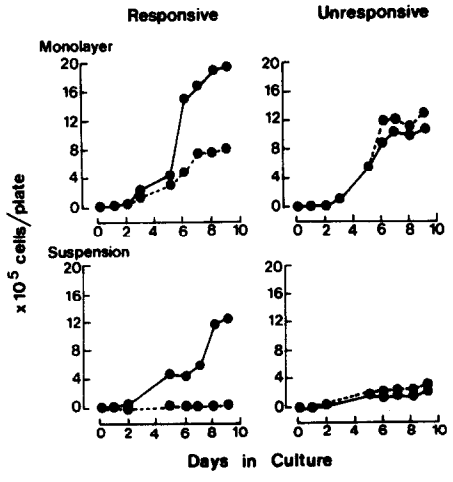

Fig. 1. Growth rates of androgen-responsive and unresponsive S115 cells in monolayer and suspension culture in the presence (—●—●—) and absence (--●--●--) of testosterone. Cells were plated at 2 × 10⁴ cells per plate.

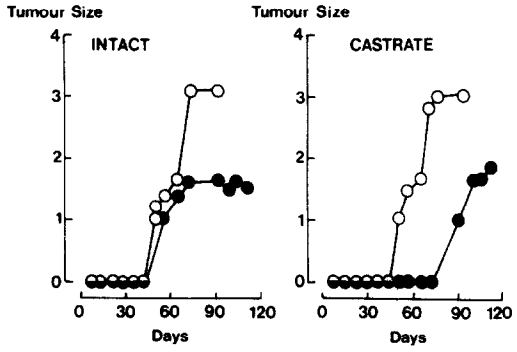

Fig. 2. Rate of tumour formation by androgen-responsive (—●—●—) and unresponsive (—O—O) cells in intact and castrated male nude mice. Tumours were scored from 1 to 4 according to size (0–2 mm, 2–5 mm, 5–10 mm, > 10 mm) and the mean score per tumour plotted.

Regrowth of tumour cells in culture

Cells from some tumours grown from androgen-responsive and unresponsive cells in intact and castrated animals were established in culture in conditions equivalent to the endocrine status of the host. After two passages in culture, the growth rate of the cells in monolayer and suspension culture was examined (Fig. 3).

Those cells which had originally been androgen-responsive and had been grown in intact mice showed a proliferative response to androgens and possessed the ability to grow in suspension culture when testosterone was present. The previously responsive cells grown in castrated mice, however, had lost the proliferative response to testosterone and were, in fact, inhibited by it. They had also lost the ability to grow in suspension culture. The cells which had originally been androgen-unresponsive remained insensitive after growth in either intact or castrated mice and did not proliferate in suspension culture.

Table 1. Rate of tumour development in nude mice

Mice	Cells	Time taken for tumour development (Days) 1st tumour	50% of mice	All mice
Intact	Responsive	49	55	> 112*
Intact	Unresponsive	49	49	55
Castrate	Responsive	89	89	> 109*
Castrate	Unresponsive	49	49	49†

*By the time of sacrifice not all of the mice had developed tumours.
†All of the mice developed tumours at the same time.

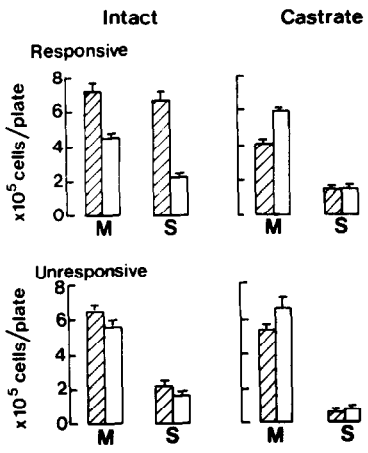

Fig. 3. Growth of androgen-responsive and unresponsive cells passaged through intact and castrated male nude mice. Cells were plated in the presence (shaded bars) or absence (open bars) of testosterone in monolayer (M) or suspension (S) culture at 2×10^5 cells/plate and counted after five days of growth.

DISCUSSION

Both the androgen-responsive and unresponsive cell types which show, respectively, 'transformed' and 'normal' characteristics in culture give rise to tumours in nude mice. In fact, the cells with the normal phenotype form larger tumours and in the case of the castrated animals the tumours appear more rapidly than those produced by the 'transformed' cells.

The rate of tumour formation in animals reflects the original androgen sensitivity of the cells. The androgen-responsive cells produce tumours more rapidly in intact animals than in castrates. Androgen-unresponsive cells, on the other hand, show identical rates of tumour formation in both intact and castrated animals.

When tumour cells are re-established in culture they retain their original characteristics with regard to testosterone stimulation and growth in suspension cultures with the exception of the previously responsive cells passaged in castrated mice, although their growth rates are slightly reduced. In the latter case the cells have lost their androgen responsiveness and ability to grow in suspension culture after a long period in an androgen-deprived environment. Thus the tumour-forming ability of the androgen-unresponsive cells cannot be attributed to some modification during passage in animals. Certainly they have not acquired anchorage independence, the parameter regarded as being most closely associated with tumorigenicity.

Clearly extreme care must be taken in equating transformed characteristics in culture with tumorigenicity *in vivo*, as has been demonstrated by others [10, 11]. These particular cell types are both derived from the same tumour and are, therefore, both technically transformed. However, their characteristics in culture are extremely different and resemble in all respects those of either normal or transformed cells. Whatever factors enable Shionogi 115 cells to give rise to tumours in nude mice, it is not the possession of transformed characteristics, including anchorage-independent growth.

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