Pergamon

European Journal of Cancer Vol. 33, No. 7, pp. 1070–1074, 1997

Printed in Great Britain
0959-8049/97 $17.00 + 0.00

PII: S0959-8049(96)00032-4

Original Paper

The Use of SCID Mice for the Growth of Retinoblastoma Cell Lines and for the Establishment of Xenografts from Primary Tumours

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We investigated the possibility of growing primary retinoblastoma tumour tissue in SCID mice. In preliminary experiments with the WERI retinoblastoma cell line injected subcutaneously in SCID mice, tumours arose at only 25% of the inoculation sites. After mixing these cells with a reconstituted basement membrane extracellular matrix (Matrigel) prior to inoculation, tumours arose at 100% of inoculated sites. When primary retinoblastoma cells were injected subcutaneously into SCID mice in the presence of Matrigel, tumours arose in 5/8 cases. On average, the latency period was 4 months before the tumours were palpable. Histopathological examination of the tumours showed that they resembled surgically resected human retinoblastomas and one of the tumours formed pseudo-rosettes which is a characteristic of these tumours. Unfortunately, when these xenografted tumours were introduced into tissue culture, it was not possible to establish cell lines directly and the cultures were soon overgrown by mouse cells which could clearly be shown to be infiltrating the tumour. The ability to grow retinoblastoma cell lines and primary tissue subcutaneously in SCID mice offers a convenient model system to study the genetics of tumorigenesis in this tumour type and possibly an opportunity to study the role of chemotherapy in the treatment and progression of the disease. © **1997 Elsevier Science Ltd.**

Key words: retinoblastoma, *in vivo* growth, SCID mice, Matrigel

Eur J Cancer, Vol. 33, No. 7, pp. 1070–1074, 1997

INTRODUCTION

ALTHOUGH THE analysis of *in vitro* characteristics of the malignant phenotype, such as loss of contact inhibition and growth in soft agar, have been useful in assessing tumorigenicity, the ability to investigate the mechanisms responsible for suppression of the malignant phenotype ultimately depends on the availability of *in vivo* models to test the effects of manipulating the cellular phenotype on tumour development. A number of animal models have been established but, for human tumours and cell lines, the nu/nu "nude" mouse system has been the most frequently used. This mutation causes the failure of thymic development, an absence of mature T-cells and impaired thymus-dependent antibody response [1]. However, despite being athymic, nude mice still demonstrate some immune competence as a result of extrathymic production of T-cells. This residual competent immune system has cast some doubt about their suitability as a host for growing primary human tumours especially for analysing suppression of the malignant phenotype. The severe combined immunodeficiency (SCID) mouse has been shown to be incapable of producing a cell-mediated immune response because of the absence of functional B and T cells [2]. Mice homozygous for the SCID mutation cannot undergo productive rearrangement of their immunoglobulin and T-cell receptor genes, which has been shown to be due to a deficiency in repairing double-strand

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Received 12 Jun. 1996; revised 25 Oct. 1996; accepted 6 Nov. 1996.

DNA breaks [3, 4]. This feature has made the SCID mouse an important *in vivo* model for the growth of human leukaemias [5–9], which consistently fail to engraft in the nude mouse. This system also offers the opportunity to expand other types of tumours, which are difficult to culture *in vivo*. This is particularly true for retinoblastomas which often fail to proliferate in tissue culture.

Retinoblastoma (Rb) is a paediatric eye tumour and has been an important model system for the study of human cancer since it has been shown that the malignant phenotype results from the inactivation of a single gene in a precursor retinal cell [10–12]. Thus, with the availability of an *in vivo* tumorigenicity assay, the ability of wild-type and mutant forms of the gene to reverse the malignant phenotype can be analysed. At present, there is some controversy about the suitability of the nu/nu mouse as a host for the Rb cells and cell lines which, some reports suggest, depends on the site of inoculation [13–15]. Thus, it appears that intra-ocular injection has a better chance of engraftment than a subcutaneous route. This is true whether nude or SCID mice are used [14–17]. However, this approach requires a high degree of skill to establish engraftment. Clearly a more simple approach, which generates successful and consistent engraftment, would be a more convenient and desirable alternative. It has been reported that primary retinoblastomas do not grow after subcutaneous injection of primary cells into nude mice [16, 17]. However, as suggested by Kleinman and associates [18], co-injection of cells with Matrigel, which includes extracts of EHS tumour cells containing laminin, type IV collagen and heparan sulphate proteoglycan promotes tumour cell growth *in vivo* [19], although its mode of action is unknown. We demonstrate here a simple method of establishing tumours from primary retinoblastoma cells and cell lines at subcutaneous sites in SCID mice by injecting the cells in the presence of Matrigel.

MATERIALS AND METHODS

Cell culture

All cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal calf serum and 2 mM glutamine. The WERI cells (a retinoblastoma cell line) only attach loosely to the tissue culture dish and were transferred by rapidly pipetting over the surface of the cells with a pipette. Human tumours grown in mice were surgically removed and chopped into small explants with curved scissors as described by Sunmerhayes and Franks [20]. These explants were seeded into 25 cm^3 culture flasks in MEM and transferred using trypsin.

Tumorigenicity testing

WERI cells were washed twice in an excess of phosphate-buffered saline (PBS) and counted using a haemocytometer. The appropriate number of cells were resuspended in 0.25 ml of PBS and injected subcutaneously into the flanks of the mice using a gauge 19 needle. Both flanks of the mice were used. Primary retinoblastoma tissue was collected within 2 h of removal from the patient, disaggregated by gentle pipetting and then frozen in MEM + 10% DMSO in liquid nitrogen. Prior to inoculation, the cells were washed in PBS and resuspended in Matrigel in a final volume of 0.25 ml. The Matrigel was stored at −20 °C, and thawed at +4°C approximately 24 h before being used. Afer resuspension of the cells in Matrigel, the cell suspension was kept on ice prior to injection. The flanks of the mice were palpated weekly to monitor tumour growth and tumours were removed before the mice showed any evidence of stress. The tumours were excised with a scalpel blade and fixed in Bouin's solution and embedded in paraffin. 0.6 μm sections were cut and stained with haematoxylin and eosin.

RESULTS

The main aim of these experiments was to determine whether we could consistently grow primary retinoblastoma cells, injected subcutaneously, in SCID mice. However, before using frozen human cells for which only small samples were available, we first tested whether the WERI retinoblastoma cell line [21], which is known to carry a homozygous inactivation of the RB1 gene [22], would produce tumours in individuals from our SCID mouse colony. There have been reports that nude mice will support the growth of WERI subcutaneously when large ($>10^7$) inocula are used. In our hands, we were unable to generate tumours in nude mice consistently, regardless of the number of cells inoculated. In the series of experiments described here, the SCID mice were all tested for human immunoglobulin production prior to inoculation and only those showing no activity were used. In a pilot study, 4 mice were inoculated on both flanks with 5×10^6, 10^7, 2×10^7 and 4×10^7 WERI cells, respectively. The cells used in the inoculum were replated into fresh tissue culture medium 36 h prior to inoculation and, at the time of harvesting, were subconfluent to ensure maximum viability of the cells. Methylene blue exclusion tests indicated that more than 70% of the cells were viable at the time of inoculation. Over a 4 month observation period, only two animals developed tumours. The first tumour arose after 4 weeks at one site receiving 5×10^6 WERI cells which was approximately $2.4 \times 1.5 \times 1$ cm in size. The second tumour arose after 7 weeks at a site receiving 2×10^7 WERI cells and was $2.6 \times 1 \times 1$ cm. The tumours grew progressively until the mice were sacrificed. Both tumours were processed for histopathology (see below). A small sample was also tested for their ability to grow in tissue culture. The tumours were soft and easy to disaggregate and progressively growing cell cultures were established after only 2 days. These cells formed a loosely attached monolayer of cells with the same *in vitro* morphology as the original WERI cells used for the inoculation. DNA was isolated from the re-established cell line and the cells were shown to be human (data not shown) by amplifying DNA sequences using PCR and oligonucleotides specific for the human Alu agent following the procedure described by Michalski and associates [23].

The incidence of engraftment of WERI, subcutaneously, in SCID mice under these conditions, was disappointingly low, although it was better than that seen with the nu/nu mouse colony available at the ICRF (London, U.K.) where we were repeatedly unable to establish tumours using a range of innoculum sizes. The reason why only certain SCID mice developed tumours is hard to explain since all the mice used were litter mates and had all been shown to be deficient in immunoglobulin production. One possibility was that the normal, loose association of the WERI cells seen on culture means that these cells are unable to aggregate sufficiently to produce a tumour subcutaneously and so attract the appropriate blood vessels and innervation from

the host. Alternatively, the subcutaneous site may not be altogether suitable for this type of cell in terms of the micro-environment which have prompted others to inoculate Rb tumour cells intra-ocularly [16]. To test whether that was important, we decided to inject the cells in the presence of an artificial extracellular matrix, Matrigel, which would presumably promote cell contact during the early part of their growth in the animal host.

Albini and associates [24] had previously demonstrated that Rb tumour cell lines can grow subcutaneously in nu/nu mice when injected in the presence of Matrigel. At 4°C the gel is liquid, but at 37°C it is solid and creates a support for cells during the early stages of tumour development. Thus, we repeated the original experiment with the WERI cell line using different inoculation sizes. Both flanks of 4 SCID mice were injected with 10^7 WERI cells and resuspended in 0.25 ml of Matrigel. After 2 weeks tumours were palpable at all eight injection sites and the tumours then proceeded to grow progressively until sacrifice of the mice after 50 days. Representative samples from three of the tumours were returned to culture and all grew progressively from the outset and showed the same *in vitro* morphology of the parental WERI cell line. Histopathological examination of the tumours showed that they were identical to those produced by WERI cells injected without Matrigel (Figure 1). Thus, in the presence of Matrigel, we have demonstrated that it is possible to achieve a highly reproducible tumorigenicity assay subcutaneously in SCID mice using a retinoblastoma cell line which grows well *in vitro*.

Many cell lines, however, are highly selected subpopulations of cells which have adapted to growth *in vitro*. To determine whether Rb cells, isolated directly from patients, would engraft in SCID mice, we inoculated uncultured tumour samples in the presence of Matrigel using the same conditions We have been collecting Rb tumours over the past 10 years as part of our analysis of the nature of the causative mutations in the RB1 gene in Rb tumours [11]. However, since the vast majority of tumours are treated successfully *in situ*, especially from hereditary cases where early screening detects relatively small tumours, it is often difficult to obtain sufficient tissue samples to attempt to establish cell lines directly *in vitro*. The problem is compounded by the fact that the number of viable tumour cells may be small since the centre of the tumours are often necrotic. In our series of tumours, all the cells were collected from enucleated eyes, within hours of their removal, and cells were frozen in 10% DMSO in liquid nitrogen, some over 10 years ago. Given the low frequency of engraftment of the WERI cells in the initial experiments, and because of the limited number of cells available for each tumour, we did not attempt to grow these cells on SCID mice without Matrigel. Single aliquots of Rb tumour cells were inoculated in the flanks of SCID mice after mixing in Matrigel. Due to lack of material in these tumour samples, cell number, or viability could not be tested. From five of the eight tumours injected, palpable tumours arose at the site of injection. After 14–20 weeks, the tumours were removed and representative samples processed for histopathology. Tumour samples which did not show evidence of necrosis were chopped into explants and put into tissue culture. Within most of these tumours were areas with a "cottage cheese" appearance which is typical of human retinoblastoma and results from calcification within the tumour.

After 4 days in culture, monolayers of cells grew but had a well-attached spindle-like morphology, presumably originating from the infiltrating mouse cells. On the surface of these cells, small round cells attached loosely which could be transferred with the fibroblast-like cells, but eventually they were overgrown by the mouse cells.

HISTOPATHOLOGICAL ANALYSIS OF TUMOURS

When the tumours from the WERI cell line, which had been injected without Matrigel, were analysed they showed undifferentiated pleomorphic cells consistent with retinoblastoma, but with no evidence of Flexner–Wintersteiner rosette formation. Also, there was evidence of necrosis within the tumour, a common feature of human retinoblastomas. Although the incidence of tumour formation for WERI cells injected with Matrigel was more consistent, the histology was similar to that seen in the WERI tumours without Matrigel (Figure 1). In both of these types of tumour, the cells showed large nucleoli with only a moder-

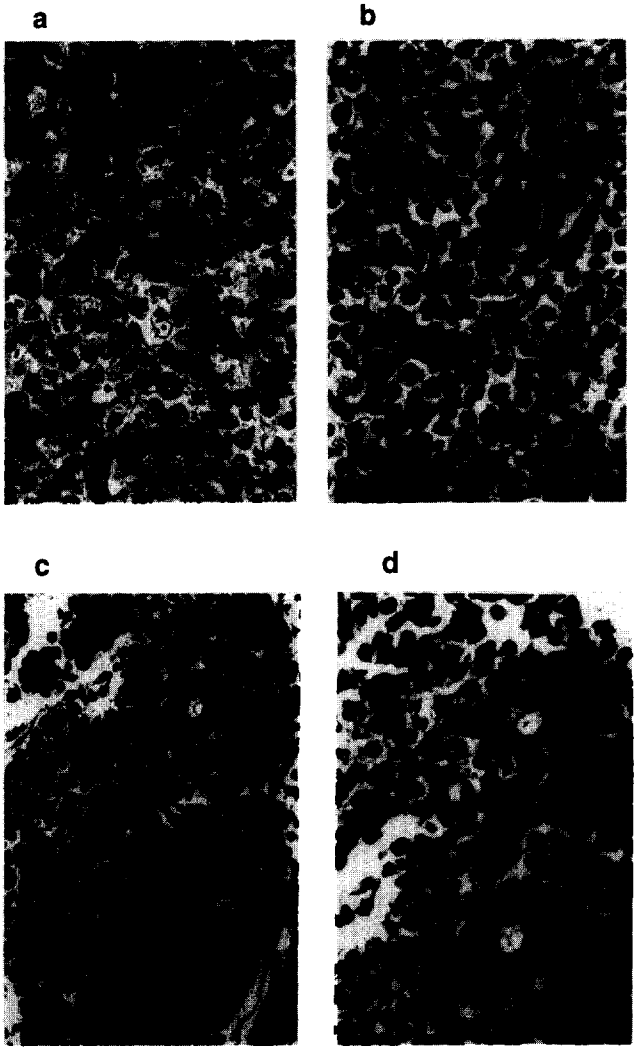

Figure 1. H & E sections of tumours arising in SCID mice following injection with (a) WERI cells in the absence of Matrigel (×400); (b)WERI cells in the presence of Matrigel (×400); (c) low-power (×100) and (d) high-power (×400) view of the tumour which arose following injection of GOS 14 cells in the presence of Matrigel. In this tumour the presence of Flexner–Wintersteiner rosettes can be clearly seen.

ate amount of cytoplasm. In four of the human tumours, 484, 769, 2147 and 45, the histology was very similar with undifferentiated pleomorphic cells (data not shown) but they were smaller and darker than WERI cells and more closely resembled surgically resected retinoblastomas, although there was no evidence of rosette formation. The fifth tumour, however, GOS 14, although resembling surgically resected tumours like the others, also showed evidence of rosettes (Figure 1). This demonstrates that the tumours formed by these Rb samples mimic the development of tumours seen in human eyes even though they were grown subcutaneously in mice.

DISCUSSION

In this study we have shown that the SCID mouse is an efficient host for primary retinoblastoma tumour cells. The tumours, if inoculated subcutaneously in Matrigel, arise consistently and within a 3–6 month period, which is important if this system is to be used in the analysis of the malignant phenotype of Rb cells. The pathology of these tumours closely resembles that seen in the human eye with rosette formation in some cases and frequent calcification. Cell lines are easier to manipulate than primary tumours because it is easier to control for cell viability and size of the inoculum. However, it is clear that injecting more than 10^7 cells does not significantly alter the incidence of tumorigenicity. However, the cryopreservation procedure used for the tumour samples was successful, but the parameters surrounding the preparation of these primary cells are harder to control. It is possible that the cells which did not grow in the SCID mice were probably not viable from the outset and examination of the inoculation site showed no evidence of tumours at all. The frequency of tumour establishment (65–70%) is comparable to that of Gallie and associates [25] who inoculated the cells intra-ocularly in nu/nu mice in order to establish growing cultures before explanting them into tissue culture. Culturing Rb cells in the eye has technical difficulties and problems with assessing tumour growth. We feel that the subcutaneous route of inoculation is far more practical. In addition, it is possible to establish far larger tumours subcutaneously which provides more material for subsequent analysis. If necessary, these primary tumours could be transplanted to increase cell numbers and with a small lag time. Albini and associates [24] showed that transplanting Y79 cells from mouse to mouse did not produce tumours in the new host in the absence of Matrigel, and so there had been no 'conditioning' of the cells during their *in vivo* passage. We have made a preliminary attempt to transfer these tumours into culture although, at present, it appears that it is the mouse cells which establish in the first instance and are probably derived from endothelial cells which are present in the infiltrating blood vessels of the tumours. It may be that including Matrigel in the cultures of the primary Rb tumours may assist in the establishment of cell lines.

Matrigel is a reconstituted basement membrane extracellular matrix extract [26]. It has been shown that co-injection of tumour cell types, including Rb, which are traditionally difficult to grow in host animals, more successfully engraft when co-injected with Matrigel. Albini and associates [24] demonstrated the growth of two established cell lines, Y79 and WERI, in nude mice using Matrigel. As with our experience, this group showed that, in the absence of Matrigel, 10^7 cells did not produce tumours after 3 months, whereas, in the presence of Matrigel, as few as 10^6 cells produce tumours in nude mice after only a short latency period. We have extended these studies to show that Matrigel has the same effect on tumorigenicity in SCID mice, but, more significantly, that primary Rb tumour tissue also produces progressively growing tumours subcutaneously in this animal model. One of these tumours (GOS 45) was derived from a patient with a germ line deletion on chromosome 13 involving bands q12–q14 [27]. In summary, the main advantage of the Matrigel–SCID system is its reproducibility, not only using cell lines such as WERI but also for primary cells which appears to recapitulate their malignant phenotype in the mouse host. After cloning of the RB1 gene, a critical series of experiments was designed to reverse the malignant phenotype by reintroducing the wild-type gene back into appropriate cell lines. In these experiments, the general impression was that the normal RB1 gene could suppress the malignant phenotype, although there was much discussion about the suitability of the *in vivo* test systems [13–15]. In some of these experiments, the ability to grow *in vitro* was not affected, whereas in others the cells were inhibited completely. Other suggestions in these early experiments was that the site of inoculation was important for being able to grow inside the eye but not subcutaneously. It is posible that the SCID mouse model described here may eliminate some of the variables encountered during these studies.

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Acknowledgement—We are grateful to the staff of the ICRF (London, U.K.) animal facility for their help in the tumorigenicity assays in nude mice.