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ORIGINAL PAPER

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The effects of meglumine gamma linolenic acid (MeGLA) on an organ culture model of superficial bladder cancer

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Abstract The objective of this research was to assess the effects of the novel intravesical drug MeGLA in a physiologically representative model of superficial bladder cancer. Petri dishes were used to culture 5 mm square explants of rat bladder in minimal volumes of supplemented culture medium. Parental and resistant MGH-U1 urothelial cancer cells were transfected with a green fluorescent protein (GFP) vector. Transfectants were purified by flow cytometry. Cells were seeded onto the prepared organ cultures and imaging was performed using confocal microscopy. Confirmation of the tumour colonies was done using scanning electron microscopy. MeGLA was added at various concentrations to the colonies and its effects noted over several days. Results showed that colonies of GFP-MGH-U1 cells established themselves on the explants and could be identified by confocal microscopy. The colonies could then be followed over several days. The colonies were able to survive high concentrations of the drug of up to 1 mg/ml, 400 times the IC > 90% for monolayers and equivalent to doses in clinical use. We conclude that MeGLA is less effective in this system than on monolayer cell lines. However, it showed cytotoxic effects which were comparable to those seen with conventional agents in the same system.

Keywords Explant · MGH-U1 cell line · Bladder cancer · GFP · MeGLA

Introduction

The essential fatty acid, gamma linolenic acid (GLA) is cytotoxic to a variety of tumour types when applied to monolayers of cancer cell lines grown in vitro [1]. The stable conjugate meglumine GLA (MeGLA) has been shown in these laboratories to be cytotoxic to urothelial carcinoma cell lines in both parental and resistant forms, at concentrations as low as 12.5 μg/ml [19]. This cytotoxicity is limited to serum free applications because of the quenching effects of protein on MeGLA. This is probably one of the reasons why trials of systemic GLA in pancreatic cancer have not shown significant efficacy [8]. However, intravesical therapy is effectively a topical application on the bladder mucosa, and therefore represents an ideal setting for the use of cytotoxic drugs in superficial bladder cancer at high concentrations, something not achievable systemically. Efficacy has been shown with monolayer cell lines, but not with more complex models.

Monolayer cell cultures have limitations because they do not simulate the multilayered cell population found in vivo, and are therefore highly sensitive to cytotoxic agents [6]. This makes it impossible to use clinically relevant concentrations for cytotoxicity assays. Attempts to overcome this difficulty have involved using animal models by induction or inoculation of tumours. However this method is unreliable in its production of superficial tumours, and their size, superficial nature and cell type cannot be controlled [13]. Thus it would be desirable to produce a model tumour system which is more reliable and amenable to experiment with cytotoxic agents at clinical concentrations.

This has been achieved by combining bladder explants grown in culture, with tumour cell lines transfected with the green fluorescent protein (GFP) gene, seeded onto the mucosal surface, in order to simulate a superficial tumour. This allows visualisation of the tumour cells under fluorescence microscopy whilst the cells remain viable, thus allowing the progress of a tumour colony to be followed over several days. The model has been tested with conventional intravesical chemotherapeutic agents and found to be consistent and able to tolerate the use of clinically relevant doses of drug [4].

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GFPs are secondary fluorescent proteins used in an energy transfer reaction to produce green light by bioluminescent coelenterates, including the jellyfish *Aequorea victoria*. The GFP cDNA encodes a 283 amino-acid polypeptide with a molecular weight of M_r 27,000, which fluoresces with a maximal excitation peak at 490 nm [2]. GFP therefore makes an ideal method of identifying tumour cells, when transfected into them by a suitable plasmid vector [10] to produce a stable GFP-expressing cell line. Multi-drug resistance is a major clinical concern in cancer treatment and leads to a lowering of cellular drug accumulation, especially in the nucleus, the main site of action of many cytotoxic drugs. Therefore, in this study both the parental and resistant clones of the MGH-U1 cell line were transfected with GFP. We have demonstrated that the transfection process does not interfere with the resistance properties of the cell lines in terms of drug uptake and that the transfection remains stable [5].

In this publicaton we describe a model using rat bladder explants and fluorescent tumour cell lines in order to simulate a superficial tumour and allow testing of MeGLA in a more physiological setting.

Materials and methods

Tumour cell lines expressing GFP

The human urothelial tumour cell line MGH-U1 was used in both its parental and multi-drug resistant forms, the latter having been produced from the parent cell line by multiple applications of mitomycin-C (MMC). It is therefore known as the MMC-MGH-U1 cell line. The original parent cell line was derived from a grade III, T2 transitional cell carcinoma, and established in 1972 [7]. It has been phenotypically characterised and maintained in these laboratories for 6 years, with regular karyotyping and the use of low passage number stock. Each cell line was maintained in Dulbecco's modified essential medium (DMEM, Sigma) containing 10% foetal bovine serum (FBS) and 1% v/v of an L-glutamine penicillin/streptomycin cocktail (Sigma). Incubation was at 37 °C in 5% CO_2 and 100% humidity.

GFP cellular transfection was achieved using the GFP vector pEGFP-N1 (Clontech Laboratories, Basingstoke, UK). The selection of transfectants was done by fluorescence activated cell sorting (FACS) using a Becton-Dickinson Vantage equipped with a 15-mW argon laser emitting light at 488 nm. The confirmation of transfection was by fluorescence microscopy. Following transfection the cell lines were maintained in culture as described above, and subcultured using trypsin–EDTA passaging [4].

Explantation

Bladders were dissected from Wistar rats killed by carbon dioxide and using a semi-aseptic technique, transported in phosphate buffered saline (PBS), and then cut into 4- to 5-mm squares. The serosal surface was glued to the bottom of 35-mm petri dishes (Nunc) using cyanoacrylate glue (Loctite). Waymouth's medium (Gibco) supplemented with a 10% FBS, L-glutamine and streptomycin penicillin antibiotic mixture (Sigma) was used to incubate the cultures with changes of medium immediately, then at 4 h, to reduce cyanoacrylate glue toxicity and daily for the first 4 days and every 2–3 days thereafter. The explants were regularly inspected by conventional inverted light microscopy for the development of monolayer "skirts" of urothelial cells.

Production of tumour explants

The GFP-labelled cell lines were passaged by trypsin-EDTA (Sigma) separation followed by centrifugation in a universal tube for 3 min at 200 *g*. The supernatant was poured off to leave a pellet of cells. The culture medium was removed from the explant petri dish using a polypropylene pipette. A hollow was created in the explant surface using the tip of an air displacement pipette into which was seeded 5 µl of cell pellet. The explant was then left to incubate for 1 h at 37 °C in 5% CO_2 without medium, in order to allow adherence of the cells to the surface. Waymouth's medium was carefully reapplied.

MeGLA

MeGLA was supplied by Scotia Pharmaceutical, Stirling, UK. Phosphate buffered saline (PBS) was used as a control as previous experiments have shown that water has cytotoxic effects. The urothelial surface was exposed to MeGLA at high concentrations visualised with acridine orange under fluorescence microscopy. Experiments on the tumour colonies were performed with MeGLA in PBS or in culture medium with 10% FCS. After removing the culture medium, 2.5 ml of MeGLA in the appropriate dilutant, at various concentrations, was applied for 1 h at 37 °C in 5% CO_2. It was then washed off and culture medium re-applied.

Confocal microscopy

Imaging was by confocal microscopy using the Leica TCS 4D point-scanning system with an argon-krypton laser emitting at 490 nm. A 5× lens was used to provide wide field images of the explant surface and GFP tumour cells before and after the application of MeGLA. Higher powers were not possible without removing the petri dish lid, and thus increasing the chances of infection. The FITC filter was used to image live GFP cells. The appropriate field was scanned up to 15 times through successive levels, with four times averaging. The series of images thus produced could be recombined to produce a single image representing a depth of 166 µm. The computer software allowed intensity measurements of a chosen field, thus enabling analysis of fluorescence before and after the application of the cytotoxic agents. However, fluorescence intensity was too variable to use as a measure of cytotoxicity, and it was more reliable to use the change in colony area. Image processing also allowed for the rotation of the series of combined confocal slices to give a transverse representation of the tumour colony.

Statistical analysis

The area of the colony on initial imaging was taken as a 100% baseline. Mean areas were calculated from four measurements of the estimated area of the colony and plotted against time. A two-tailed Student's *t*-test was used to compare pairs of values and significance was set at $P < 0.05$.

Bromodeoxyuridine staining

A bromodeoxyuridine (BrDU) staining kit (Boerhinger Mannheim, Germany) was used to stain those cells in S phase. The technique was first established on monolayer cultures followed by the urothelial surface of the explant and finally used to verify cell turnover on the GFP MGH-U1 colonies. The explant tumours were incubated with BrDU for 30–480 min, and then fixed in 70% ethanol in glycine buffer pH 2 for 20 min. Mouse anti-BrDU primary antibody incubation for 30 min was followed by TRITC-conjugated anti-mouse secondary antibody for 30 min. The resulting specimen was then viewed using the techniques described above.

Results

Control experiments

The explant tumours grew at a steady rate without the addition of any drug and similarly showed no inhibition by PBS (Figs. 1, 2). There was, however, some variability in the growth of the colonies, as indicated by the wide error bars in the graphs.

Water did have a significant cytotoxic effect, either leading to the complete destruction or a significant reduction in colony size (Fig. 3). These effects were seen within 3 h of exposure, after which the colonies continued growing. This served as a useful test of the system and for comparison with drug exposure.

BrDU staining

When GFP-MGH-U1 cells were stained, the GFP fluorescence was quenched by the ethanol fixative, and alternative fixatives did not solve this problem. Thus, when the GFP colonies were stained the pre-stained colony was imaged and mapped before BrDU staining of the colony. This demonstrated that a proportion of the cells stained positive to BrDU (Fig. 4), although the technique did not allow for accurate quantification.

Sensitive MGHU-1 colonies

MeGLA in PBS was used (Fig. 5) and over a period of 3 days showed significant destructive effects only at the

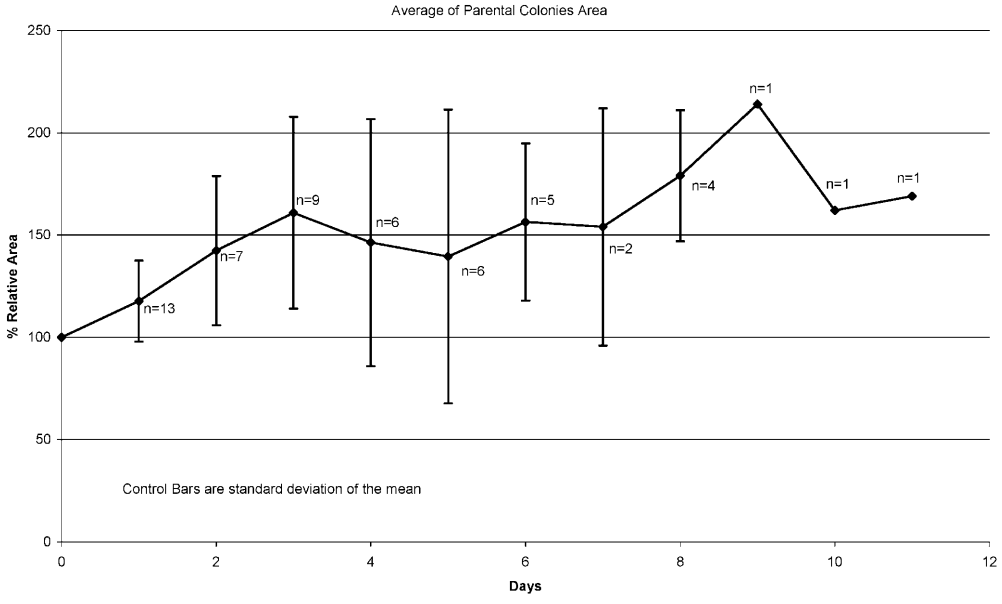

Fig. 1. The mean growth of parental MGH-U1 colonies

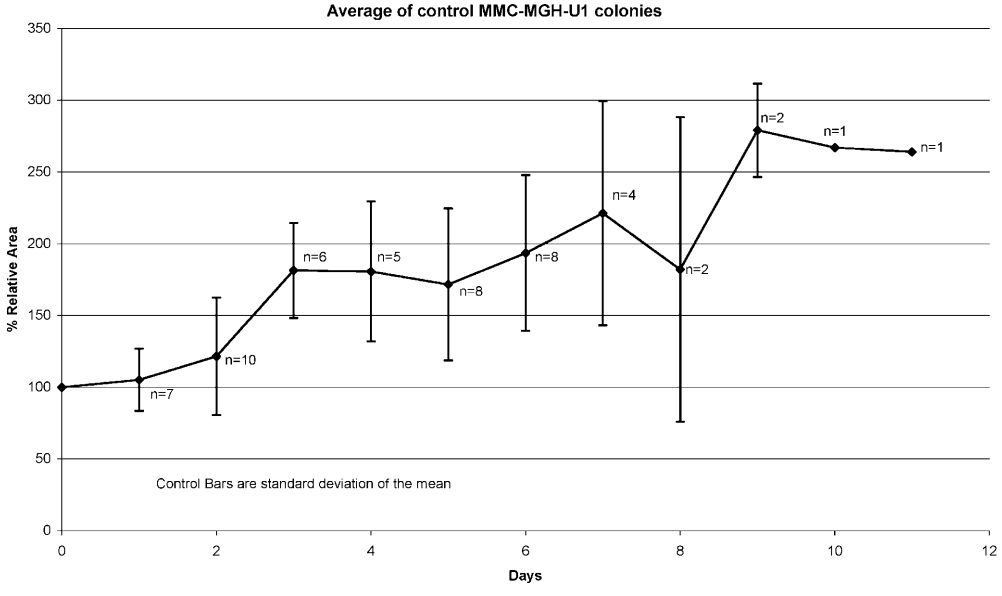

Fig. 2. The mean growth of mitomycin-C (*MMC*)-resistant MGH-U1 colonies

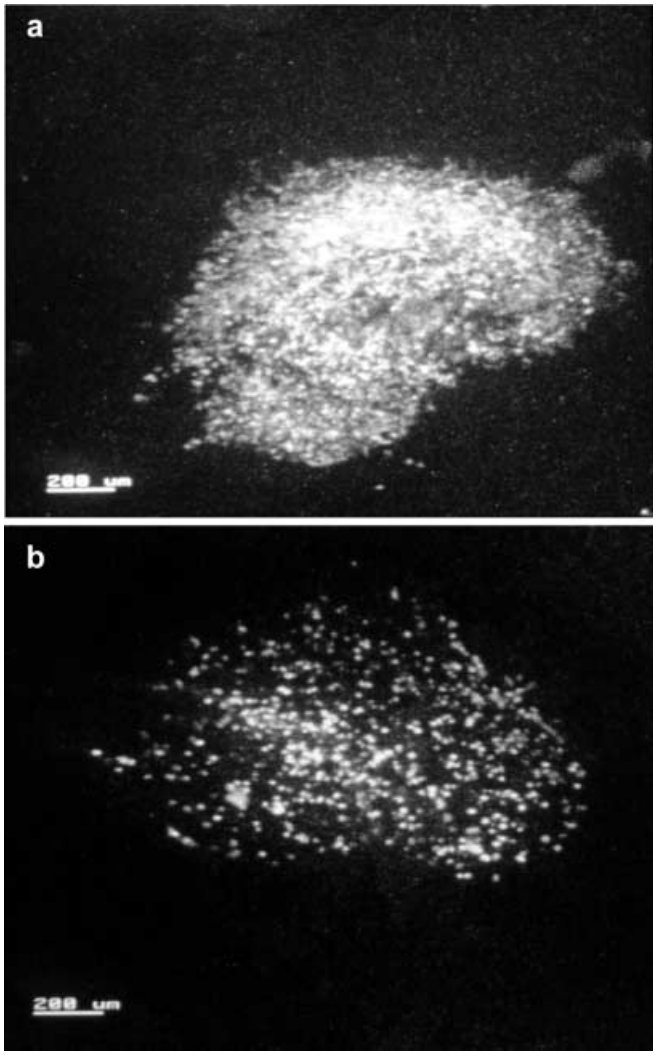

Fig. 3a, b. **a** Green fluorescent protein tumour exulant colony 1 day after seeding, **b** 24 h after application of water for 1 h

highest concentration of 1 mg/ml. This was seen at the early stages, 2 h after exposure. The colony area then began to recover over the ensuing days. These results were significant with $P < 0.005$ for all comparisons with 1 mg/ml.

MMC resistant MGHU-1 colonies

MeGLA in PBS again showed similar results to the parental cells over a period of 4 days (Fig. 6). However, in this case no fluorescence was seen after 3 days at the high concentration of 1 mg/ml. The other colonies continued to grow, without any appreciable effect on them. A statistical significance of $P < 0.005$ was found for comparisons between 1, 10 and 100 µg/ml. No comparison was possible for the highest dose of 1 mg/ml as no cells remained after drug exposure. The experiments performed with MeGLA dissolved in culture medium with 10% FCS showed that the colonies grew at

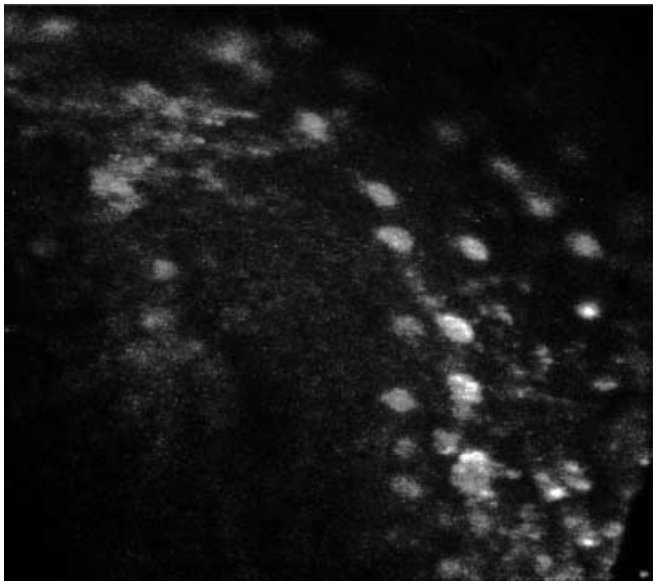

Fig. 4. BrDU stained GFP MGH-U1 colony cells in S phase demonstrating active proliferation

a similar rate for the lower concentrations (Fig. 7). Some effect was initially seen at 1 mg/ml, but the colony continued to grow thereafter. $P < 0.005$ was found for all comparisons with 1 mg/ml, except at 3 days for 100 v 1 mg/ml ($P = 0.27$).

Discussion and conclusions

These results show that the tumour colonies can survive high concentrations of MeGLA similar to those in clinical use, and also enable the same tumour colony to be followed over time. The development of monolayer skirts from the explant cultures themselves shows that the urothelium is living and capable of migration and growth, and corresponds to our previous observations on explant culture [4]. The experiments with MeGLA on the explant per se further showed that the urothelial surface is able to withstand a toxic insult without denudation of the epithelial surface. The characteristics of these explants are similar to those described previously [17] with a similar division of the explant into normal surface urothelium and epithelial-like growth on the surrounding base [21]. The behaviour, conditions for culture and morphology of these explant systems are, therefore, well established. The primary method of examining these models has been by conventional histology or by SEM, whereas this system has the advantage of being able to view the living tumour colonies without terminating the experiment. However, frequent removal of the cultures from the incubator increases the chance of microorganism contamination and effectively limits the period for which the explants are maintained to about 20 days. This compares to other studies in which the urothelium has been maintained in culture for up to 200 days [12].

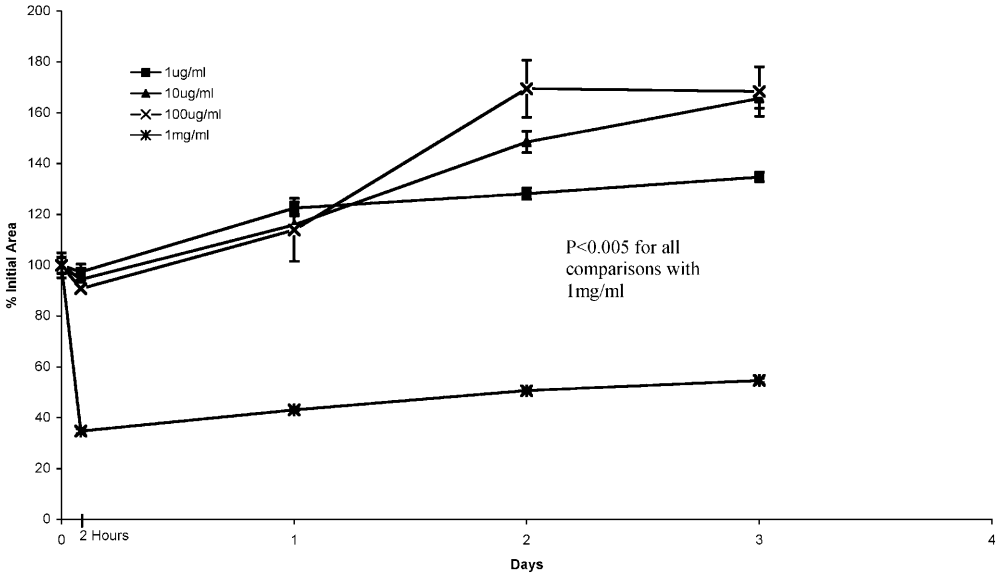

Fig. 5. The effects of a 1 h exposure to MeGLA at various concentrations on parental MGHU-1 colonies. The area of the colony is expressed as a percentage of the pre-exposure state and *error bars* are the standard deviation of the mean of four estimates of the colony area

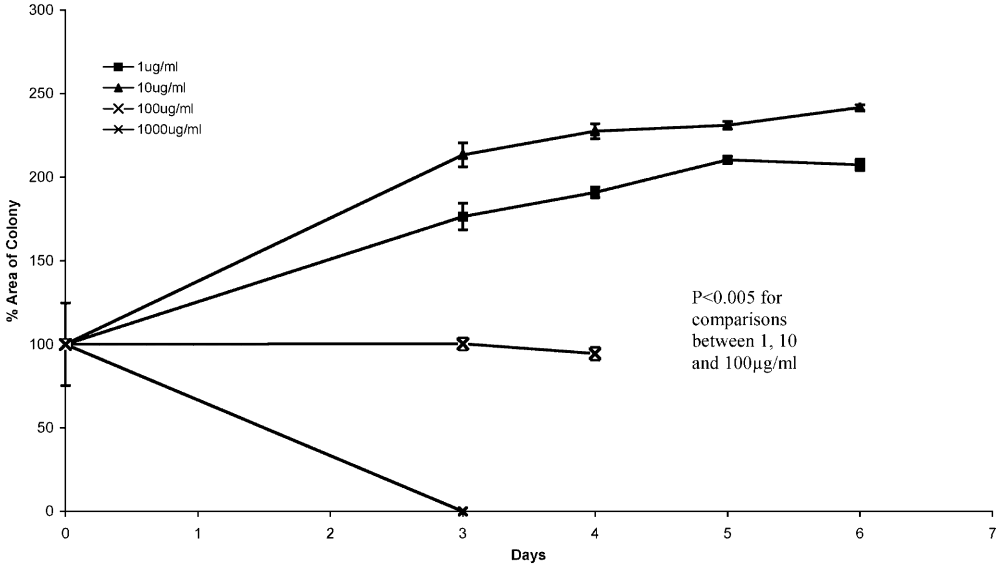

Fig. 6. The effects of a 1 h exposure to MeGLA at various concentrations on mitomycin-C (*MMC*)-resistant MGHU–1 colonies. The area of the colony is expressed as a percentage of the pre-exposure state and *error bars* are the standard deviation of the mean of four estimates of the colony area

The SEM images confirmed the presence of tumour cells in a defined colony that correlated with the confocal images and showed the morphology of the surrounding urothelium. Their roughened appearance and larger size could readily identify the cancer cells. Stranded processes between the tumour cells and urothelium suggested that junctional bonding had occurred. Previous studies using SEM have shown similar images of the normal and hyperplastic surface [11, 21]. BrDU staining is an established method to demonstrate DNA replication [9]. BrDU staining of the explant colonies showed a proportion of cells staining positive, suggesting that these cells were in S phase. This indicates that the colony is capable of proliferation, although it was not possible to accurately quantify this because of difficulties in synchronously staining both non-S phase and S phase cells.

The advantage of using a fluorescent tumour cell line inoculated into normal urothelium is that it provides a patho-physiological imitation of the in vivo superficial bladder tumour. Potential therapeutic agents may, therefore, be investigated in a manner that will relate better to the clinical scenario by allowing clinically relevant concentrations of drugs to be tested. The fluorescent nature of the transfected tumour cells allows the visualisation of the tumours without recourse to histological examination. It also demonstrates their viability.

The fluorescence intensity analysis was too variable for use as a cytotoxicity measure because of difficulties in calibration and the way in which the computer software interpreted the data. There was also some background fluorescence to be taken into account and condensation on the petri dish lid varied from day to day. Estimates of

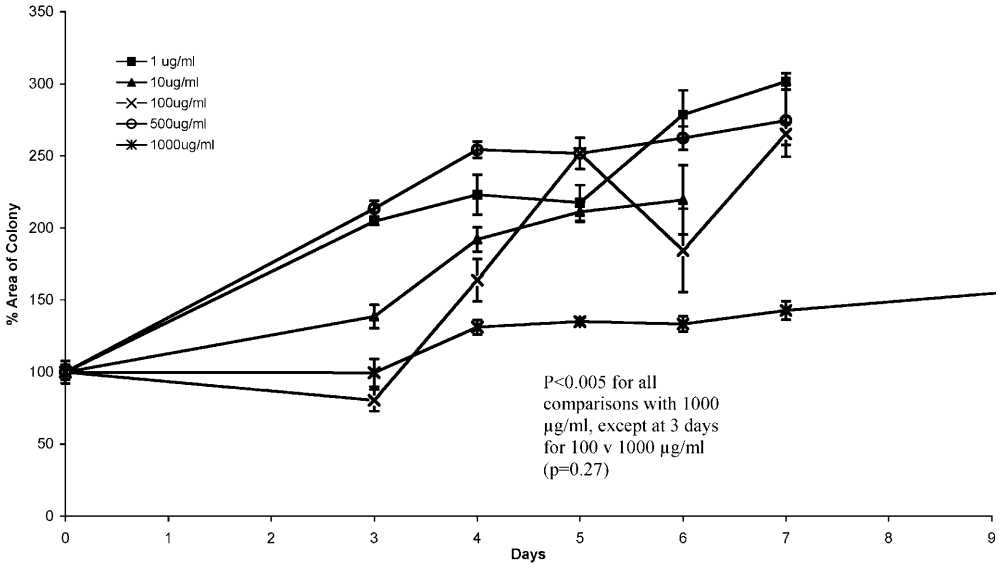

Fig. 7. The effects of a 1 hour exposure to MeGLA in medium with 10% FCS at various concentrations on MMC resistant MGHU-1 colonies. The area of the colony is expressed as a percentage of the pre-exposure state and error bars are the standard deviation of the mean of 4 estimates of the colony area

cytotoxicity were by measurement of the area of a colony. This gave a more accurate reflection of the effects of an applied drug. This technique is more time-consuming than colourimetric assays on monolayer cell lines, which results in smaller data sets that take longer to collect, although the results are more relevant to potential in vivo effects.

The control experiments demonstrated that the colonies will grow at a steady rate. The slower growth of the parental cells probably represents the variable nature of different colonies, as well as the error in measurement inherent in the technique used. Water has previously been shown to have cytotoxic properties and so the effects seen with the explant colonies were as expected [20].

Parental "sensitive" tumour cells survived a high dose of 1 mg/ml of MeGLA and then recovered to continue growing. Future experiments will be used to assess the ability of the model to undergo repeated exposures to cytotoxic agents, including MeGLA. The colonies exposed to lower concentrations behaved in a similar fashion to control colonies. In comparison, the MMC-resistant cells were destroyed 3 days after exposure to MeGLA at this dose, but appeared relatively unchanged after exposure to 100 µg/ml. These results suggest that a much higher concentration is indeed required for tumour killing than is suggested by experiments with monolayer cell lines. This has clinical relevance in terms of assessing the most effective dose to use. The tumour cells may be protected by their attachment to normal urothelium or the multilayered colony may prevent full penetration of the drug [23]. In this study the explant colonies were able to withstand a dose of MeGLA ten times the previously observed IC50. It was not possible to calculate an equivalent IC50 because of the different technique used to assess tumour colony growth. The experiments with FCS confirmed the observations of previous studies [18]. There was some inhibition at the highest concentration with the colony remaining the same size. At the lower concentrations no effect on growth was seen.

Yeast and other fungal infections were a constant problem, probably because serial imaging necessitated daily removal of cultures from the incubator to a distantly located confocal microscope. This was the reason why cultures could not be maintained longer than about 20 days. Human and animal tissue combined is the most practical solution to the problem of obtaining a regular supply of fresh human tissue and producing a rational series of experiments using a standard technique.

There have been previous attempts at combining tumour cell lines and normal tissue. For example, when an artificial extracellular matrix was used with tumour cells, firm attachment of cells occurred within 1 h [14]. The interaction between superficial tumour with normal bladder stromal cells showed that there was no stimulatory effect on growth, but cells derived from an invasive cell line were strongly stimulated [15]. Spheroids are an alternative complex model. Their effect on bladder explants has been assessed for invasiveness in the presence of growth factors [22]. An in vitro model has also been designed in which mouse tumour cell lines of various grades were inoculated adjacent to mature urothelial explants [16].

This system is unique in allowing high concentrations of cytotoxic agents to be used and their effects observed over time. It has shown that MeGLA is not as potent as monolayer cultures would suggest, but at high concentrations it still has destructive effects on tumour colonies. These experiments suggest that the highest doses are needed clinically to produce a response. MeGLA is well-tolerated intravesically at doses up to 2.5 mg/ml [3]. A phase I study showed good toleration of intravesical MeGLA [3] and a phase II efficacy study is currently recruiting.

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