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Report

Selective cytotoxicity of gemcitabine in bladder cancer cell lines

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We have examined the cytotoxic effect of gemcitabine in intravesical therapy using an *in vitro* co-cultured spheroid model composed of transitional cell carcinoma (TCC) and fibroblasts from both human and rat species. Immunohistochemistry analysis of the co-cultured spheroids, using cytokeratin-13 and vimentin antibodies against TCC and fibroblasts, respectively, showed the central location of fibroblasts within the spheroid, whereas TCC formed the peripheral layers. Spheroids composed of human TCC and fibroblasts (MGH-U3/CRL-1120 or RT-112/CRL-1120) as well as rat TCC and their corresponding fibroblasts (AY-27/RF-Ed1) displayed the same drug tolerance profile after an exposure of 0, 1, 3, 5, 7 and 14 days. As confirmed by time-lapse photography, MTT essay and vital dye staining, gemcitabine selectively killed the human and rat bladder cancer cell lines, but did not affect un-transformed human and rat fibroblast lines. [© 2002 Lippincott Williams & Wilkins.]

Key words: Gemcitabine, intravesical chemotherapy, spheroids, transitional cell carcinoma.

Introduction

Superficial bladder cancer (TNM classification: Ta, Tis or T1) represents 70–80% of the 4400–5000 new cases of bladder cancer diagnosed in Canada and 53 000 diagnosed in the US annually. Recurrence of superficial tumors is frequent; being as high as 80% within 5 years and thus necessitates frequent surveillance. Progression to muscle invasive disease may occur in 5–20% of Ta tumors, 13–75% of T1 tumors and 60% of Tis tumors.[1,2] Currently, adjuvant intravesical Bacillus Calmette-Guerin (BCG) is considered the best treatment option following transurethral resection. BCG is believed to work by indirect stimulation of the immune system.[3] Being an attenuated *Mycobacterium*, BCG carries the risk of infection including systemic infection (BCGosis), severe cystitis and prostatitis. An ideal therapy for superficial bladder cancer would selectively destroy only the transformed cancer cells, with no adverse reactions or injury to normal tissue. To this ends, we have examined the cytotoxic effect of gemcitabine for intravesical therapy using an *in vitro* assay of co-culture spheroids composed of human TCC cell lines MGH-U3, RT-112, rat TCC cell line AY-27, and human or rat fibroblasts CRL-1120 and RF-Ed1, respectively. Time-lapse photography, vital dye staining, and clonogenic and MTT assays were employed to detect selective killing of the transformed cells.

Gemcitabine [2′,2′-difluorodeoxycytidine (dFdC)] is unique among nucleosides in that it is effective in the treatment of a broad spectrum of cancers of epithelial origins including breast, non-small cell lung, pancreatic and ovarian cancers.[3] Substantial synergism with other drugs is one of the most important characteristics related to dFdC. Indeed, it has been shown that dFdC possess a striking clinical activity in the systemic treatment of advanced urothelial cancers, both as monotherapy[4–6] and in combination with other agents.[7,8]

Several characteristics of dFdC make it a promising candidate for intravesical therapy of superficial bladder cancers. Since dFdC is a non-vesicant drug with little local toxicity, high drug concentrations can be achieved in the bladder without the expectation of

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drug-induced cystitis.[9] Unlike mitomycin C, dFdC substantially penetrates the bladder mucosa, making possible the intravesical treatment of early invasive disease.[9] It is not clear whether dFdC preferentially produces cytotoxicity in bladder carcinoma cells, while sparing the non-transformed bladder mucosa and submucosal tissues.

In the present study, we report on the substantially selective cytotoxicity of dFdC in an *in vitro* three-dimensional bladder carcinoma model. Selective killing of the human cancer cell lines MGH-U3 and RT-112, as well as the rodent TCC line AY-27 was observed, whereas normal human and rat fibroblasts, cultured alone or with TCC as a spheroid co-culture, did not display any cytotoxic effects of dFdC.

Methods

Cell culture and spheroid preparation

Human TCC cells MGHU3 and RT-112,[10,11] as well as human and rat fibroblasts were grown routinely as monolayers in DMEM medium (Gibco/BRL, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS; Gibco/BRL) at 37°C in a humidified atmosphere of 5% CO_2. The rat TCC (AY-27) line[12] was grown in RPMI 1640 medium supplemented with 10% FBS at 37°C and 5% CO_2. A liquid overlay (semisolid) culture technique was used to produce multicellular spheroid culture (MCS)[13] as follows. Monolayers were detached using 0.01% trypsin (Gibco/BRL) in PBS/EDTA. Cells were then suspended in growth media and their number determined using a Coulter counter (Coulter, Burlington, Ontario, Canada). Cells were seeded at a concentration of 3000 cells/well of 96-well plates for tumor cells and 6000 cells/well for fibroblasts in 200 μl of medium onto 100 μl of solid 1% agarose. After 3 days of incubation, single MCS had formed. Each fibroblast MCS was transferred with a pipette tip into a well with a tumor MCS. Co-cultures were ready for experimentation after 3–4 days. Fibroblasts provided the optimal substrate to generate an *in vitro* spheroid model that could tolerate cooperative growth with malignant cell lines.

Immunohistochemistry staining

Formalin-fixed, paraffin-embedded spheroid sections were immunohistochemically stained using standard methods.[14] Since each co-culture spheroid contained a TCC epithelial line and a fibroblast line, TCC-specific anti-cytokeratin 13 (clone CAM, 5.2) (BD Canada, San Jose, CA) and fibroblast-specific anti-vimentin (Dako, Mississauga, Ontario, Canada) antibodies were used, respectively, to differentiate the organization of cellular components within the MCS. Sections (4 μm) were cut and mounted on Superfrost Plus microscopic slides (Fisher, Nepean, Ontario, Canada), and placed in preheated 1 mM EDTA solution (pH 8.0) before 10 min of microwave heat at 500 W. The slides were then washed for 5 min in warm running water and subjected to the ABC detection kit procedure.[15] Briefly, the slides were incubated in 3% H_2O_2/methanol for 10 min to block endogenous peroxidases, then incubated in a blocking solution composed of 75 μl of goat serum in 5 ml of PBS for 30 min. The primary antibody was added for 30 min at room temperature, followed by a 5 min wash in PBS/0.1% Tween 20 and then incubated with biotinylated secondary antibody solution at room temperature for 20 min. A washing step of 3 × 5-min each was performed in PBS/Tween 20 before incubation with streptavidin reagent at room temperature for 20 min. The slides were washed as indicated before in PBS/Tween 20 and revealed in peroxidase substrate (diaminobezidine). After 15 min wash in running tap water, slides were incubated in 1% copper sulfate for 3 min followed by a brief wash in water. The slides were subsequently counter-stained in hematoxylin, dehydrated in alcohol and mounted with Permount after a 5-min wash in xylene. Pictures were prepared using a Leica DMRE microscope equipped with a Cooke/Semsicam camera.

Gemcitabine treatment

Cell monolayers and spheroid co-cultures were continuously exposed to gemcitabine at different concentrations of 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M in media, and incubated at 37°C as described above. Trypsin-treated monolayer cells were harvested after variable time intervals and single cells plated for clonogenic assays. In order to determine growth inhibition, cells were exposed to gemcitabine in 96-well plates before a standard MTT assay was applied. The cytotoxic effect of gemcitabine was estimated by monitoring spheroids in culture at days 0, 1, 3, 5, 7 and 14 under a Zeiss Axioplan II inverted microscope equipped with a digital camera generating time-lapse images.

Viability testing

Gemcitabine-treated spheroids and controls were stained at days 0, 3, 7 and 14 using live cell nucleic

acid stain Syto16 (Molecular Probes, Eugene, OR) and propidium iodine (PI) (Sigma St Louis, MO). Syto16 and PI were used at the final concentration of 10 and 5 μM, respectively.

A combined solution, made of both dyes, was added at the described times after gemcitabine treatment to the Lab-Tek II chambered coverglass (Nalge Nunc, Naperville, IL) containing agarose gel and spheroids (co-cultured or single) and incubated at 37°C for 1 h. Spheroids were then viewed and optical thin slices were generated through a confocal microscope (Zeiss LSM510) with the following filter characteristic combination: for Syto16: excitation/emission=488 nm/BP505–550 nm and for PI: excitation/emission=543 nm/LP560 nm.

Quantification of cell viability

Confocal images were analyzed by using the MetaMorph software program, which permits quantification of emitted light from each image at an appropriate section. Images were taken using a Zeiss Axioplan II inverted microscope equipped with a CCD digital camera and a computer that serves for both computing and video-capturing images. Shortly, the Metamorph software acquires a digital image through the CCD camera that has been set up for the best exposure time. The software enumerates pixels for alive and dead cells (green and red fluorescent color respectively), and the percentage of live cells is calculated by the ratio of these two values.

MTT assay

A modified version of the MTT growth and survival assay originally described by Mosmann in 1983[16] was utilized. Briefly, monolayer exponentially growing cells were harvested using 0.01% Trypsin/EDTA and plated into 96-well plates at 20 000 cells/well. Plates were incubated overnight at 37°C in 5% CO_2 atmosphere. Cells were then continuously exposed to a range of concentrations of gemcitabine (10^{-7}–10^{-4} M) and incubated at 37°C for 3 or 7 days prior to MTT assay. Controls were cultures that were treated in media alone. At the stated time, the media was removed from cultures and cells were washed with warm PBS (pH 7.4). An aliquot of 50 μl of the MTT solution made of 0.5 mg/ml in serum-free media was added to each well, before an incubation of 4 h at 37°C. Then, 100 μl of 0.04 N acid (HCl) isopropanol (100%) was added to dissolve any converted formazan crystals. Plates were then gently agitated on a shaker at room temperature to allow complete dissolution of all crystals and read using an ELISA plate reader (BioRad Benchmark, model EL 800) at a dual wavelength of 570 and 650 nm for test and control, respectively.

Clonogenic cell survival assay

Rat TCC (AY-27) cells from exponentially growing cultures were harvested using 0.01%. trypsin in PBS/EDTA at 37°C and centrifuged for 5 min at 75 *g*. Cells pellets were resuspended in RPMI 1640 containing 10% FBS and seeded at density of $1.5–2.5 \times 10^5$ cells/35-mm Petri dish. Then incubated overnight at 37°C and 5% CO_2. Cells were subsequently exposed to increasing concentrations of gemcitabine (10^{-7}–10^{-4} M), and incubated at 37°C for incremental periods of 0, 1, 3 and 6 h. Then they were washed in PBS/EDTA and harvested using 0.01% trypsin in PBS/EDTA and the single-cell suspensions were counted. Known numbers of cells were plated in 60-mm Petri dishes and incubated at 37°C for 1 week. Each dose was repeated in triplicate for at least three independent experiments. The colonies were stained with methylene blue and counted.

Results

Immunohistochemical characterization of co-cultured spheroids

Co-cultured spheroids composed of TCC and fibroblasts were organized in central and peripheral entities that could be easily discriminated under standard light microscopy. In order to ascertain the growth orientation of cells (RT-112 and CRL-1120) co-cultured as spheroids, immunohistochemistry technique was applied. Frozen sections (8 μm) of fixed spheroids were cut and mounted on Frost Plus microscopic slides. Antibody directed against cytokeratin 13 and vimentin recognizing, respectively, epithelial cells and fibroblasts were applied. Figure 1(A and B) displays the central position of fibroblasts (CRL-1120) (vimentin-positive cells) and the peripheral orientation of RT-112 TCC (cytokeratin13-positive cells).

Time-lapse photography of TCC and fibroblast co-cultured spheroids

Time-lapse photography of co-cultured spheroids composed of TCC cell lines and their corresponding

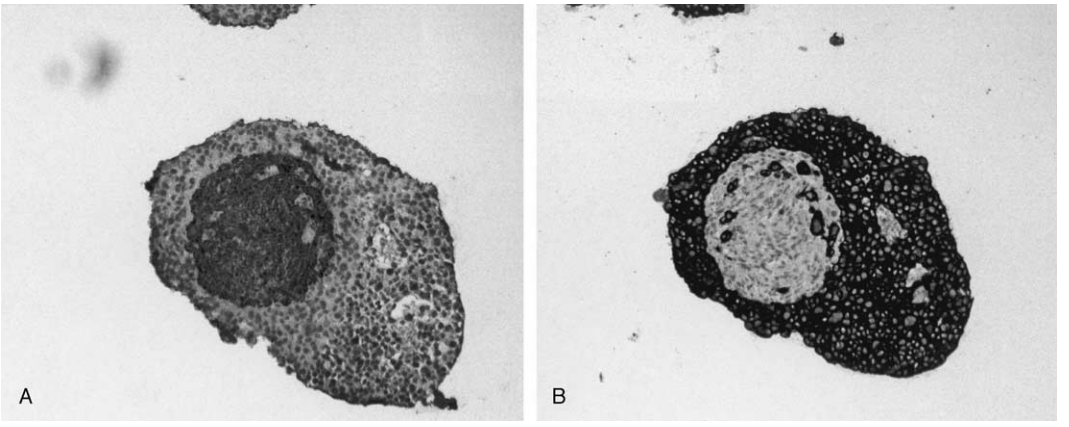

Figure 1. TCC and fibroblast localization in co-cultured spheroids. Co-culture spheroids (RT-112/CRL-1120) were stained for cytokeratin 13 (epithelial cells) (A) or for vimentin (fibroblasts) (B), revealing a peripheral orientation for RT-112.

fibroblasts (MGH-U3/CRL-1120, RT-112/CRL-1120 and AY-27/RF-Ed1) was used to monitor the cytotoxic effect of gemcitabine. Spheroids were exposed to gemcitabine (10^{-4} M) for varying periods of time (0, 1, 2, 3, 5, 7, 14 and 21 days) and compared to untreated controls.

Time-lapse images of MGH-U3/CRL-1120 spheroids show that over a span of 3 weeks, control spheroids grew and remained healthy, while the gemcitabine-treated spheroids displayed a clear disruption of their integrity and structure (Figure 2A). However, when spheroids were composed of fibroblasts alone, no difference in growth and structure was observed between gemcitabine-treated and untreated spheroids (Figure 2B).

The gemcitabine selective killing of TCC was further shown on spheroids composed of RT-112 TCC and CRL-1120 fibroblasts. Likewise, a selective disruption effect of gemcitabine on the RT-112 altered the normal growth pattern of the RT-112/CRL-1120 co-culture spheroid (Figure 3A and B). Similar results were obtained when rat TCC cell line (AY-27) and rat fibroblasts (RF-Ed1) were co-cultured as spheroids and exposed to gemcitabine (data not shown).

Vital dye staining

To confirm the above observations on selective killing of tumor cells by gemcitabine, we used a vital dye technique in which live and dead cells are discriminated according to the uptake of different colored dyes. Live cells have affinity to Syto16 dye that emits green fluorescence at 550 nm, whereas dead cells uptake PI and will emit red fluorescence at 560 nm.

Spheroids composed of MGH-U3 cells and CRL-1120 fibroblasts were exposed to 10^{-4} M gemcitabine for 0, 3, 7 and 14 days, and monitored for the time dependence of cytotoxic cell killing. For homogeneous spheroids made of exclusively MGH-U3 cells (Figure 4A and B), the number of MGH-U3 cells taking up PI substance (dead cells) increased with time in gemcitabine-treated TCC spheroids (Figure 4B) when compared to untreated controls (Figure 4A). Similarly, in heterogeneous cultured spheroids (MGH-U3/CRL-1120, Figure 4C and D), the PI uptake (red in the original images) was also time-dependent for TCC cells, whereas CRL-1120 fibroblasts took up Syto 16 (green in the original images), suggesting tumor-cell-specific gemcitabine cytotoxicity. CRL-1120 spheroids, gemcitabine-treated or not (Figure 4E and F), displayed the same profile of cell viability either as a homogeneous cultured spheroid (Figure 4E and F) or in co-culture with MGH-U3 TCC cells (Figure 4D and C). Spheroids composed exclusively of RT-112 TCC cells and exposed to gemcitabine over the period of 0, 3, 7 and 14 days displayed the same TCC killing as seen in GMH-U3 TCC cells (Figure 4G and H).

Quantification of cell viability

Confocal images taken from gemcitabine-treated spheroids and non-treated controls made of MGH-U3 cells alone (Figure 5A), MGH-U3/CRL-1120 (Figure 5B), fibroblasts alone (Figure 5C) and RT-112 alone (Figure 5D) were transferred to the

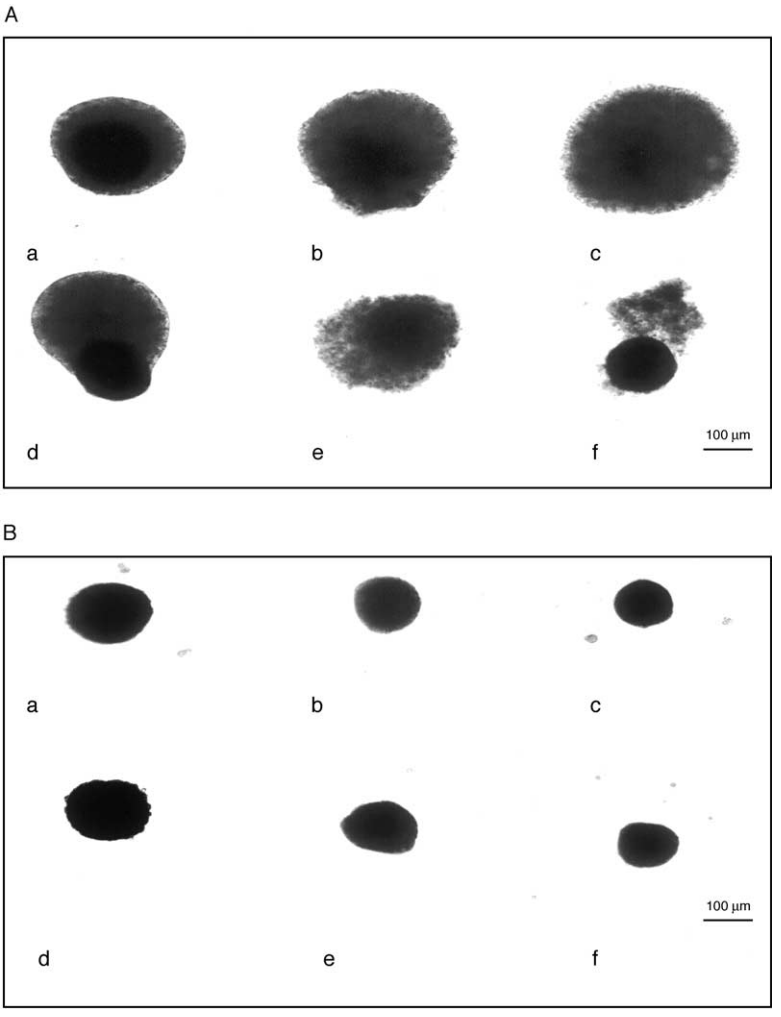

Figure 2. Selective gemcitabine cytotoxicity of co-cultured spheroids. Spheroids composed of MGH-U3 and CRL-1120 cells (A) were treated with gemcitabine. Time-lapse images were produced, and compared from both control (Aa–c) and gemcitabine-treated spheroids (Ad–f) at days 0, 7 and 21. Single composed spheroids (CRL-1120 alone) were treated with gemcitabine (B). Controls (Ba–c) as well as treated spheroids (Bd–f) over the same period of time are shown.

Metamorph software program for quantification. Green light emitted from live cells and red light from dead cells were quantified and compared. In the case of homogeneous TCC spheroids, more than 70% of the initial number of cells were dead after 3 days of exposure (Figure 5A and D). At 14 days of gemcitabine exposure, only a percentage at the level of background was observed (less than 5% of live cells), suggesting the complete destruction of TCC cells forming the spheroid by gemcitabine in 2 weeks time. In the co-cultured spheroids (TCC/fibroblast) (Figures 5B), more than 40% of the TCC cells vanished after 3 days of exposure, whereas 14 days exposure was fatal for at least 95% of the population of TCC cells. Fibroblasts composing the same heterogeneous spheroid and subjected to the same conditions of gemcitabine exposure remained viable (Figure 5B). This is clearly illustrated in Figure 4(D)—the killing of TCC cells located around the fibroblast at days 3, 7, and 14 of the treatment is achieved, whereas the fibroblasts remain intact. In cultured spheroids composed exclusively of fibroblasts (Figure 5C), the high percentage of living cells showed no significant differences between gemcitabine exposed and non-exposed fibroblasts. In both cases, spheroids tested after 14 days treatment were similar to control in shape, size and dynamic character of living cells.

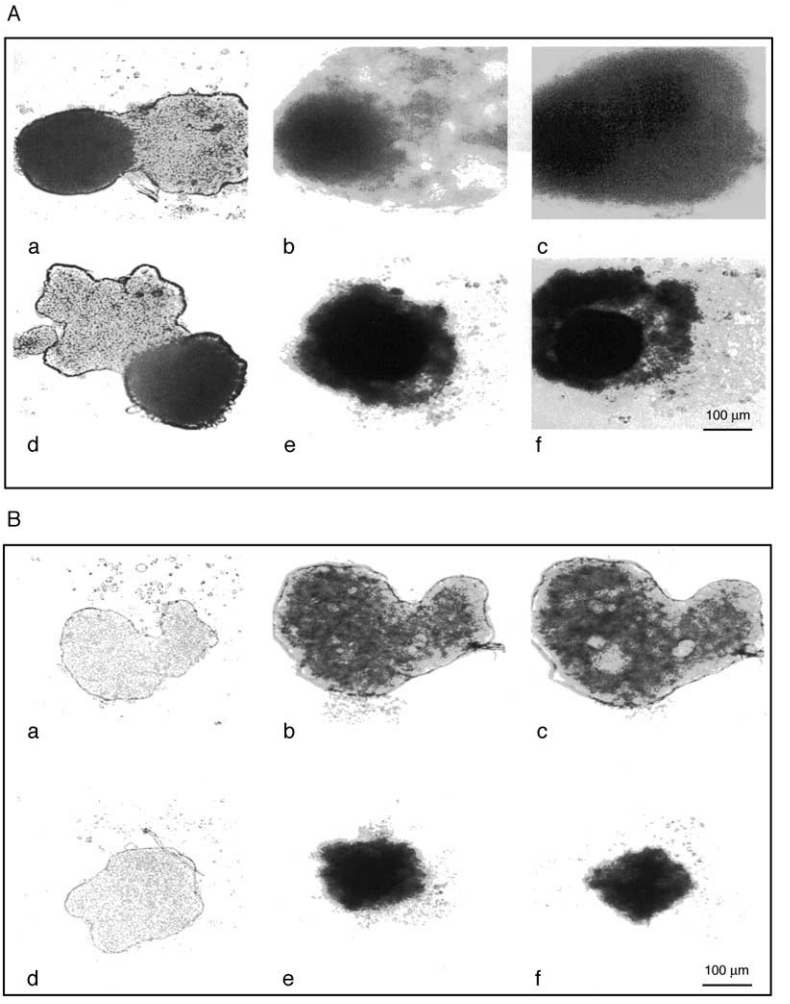

Figure 3. Selective gemcitabine cytotoxicity of co-cultured (RT-112/CRL-1120) and pure (RT-112) spheroids. Time-lapse images of control (Aa–c) and gemcitabine-treated (Ad–f) co-culture spheroids at days 0, 7 and 14 are shown (A). (B) Single composed spheroid (RT-112 alone) images were taken at the same time intervals as co-culture spheroids shown in (A). (Ba–c) Controls and (Be–f) treated spheroids.

MTT assay

Differential killing of various cell lines was assayed by MTT cytotoxicity assay. Plated cells (MGH-U3, RT-112, AY-27 and CRL-1120) were continuously exposed to incremental concentrations of gemcitabine (10^{-7}–10^{-4} M) and incubated for either 3 or 7 days. At the appropriate time point, MTT assay was performed to compare the cytotoxic effect of gemcitabine on the various cell lines. Rat TCC cell line AY-27 was the most sensitive among these lines, whereas fibroblasts CRL-1120 were the least sensitive. AY-27 cells showed 1.5-log kill when incubated for 3 days at the highest concentration of 10^{-4} M (Figure 6) and that remained the same after 7 days of incubation (data not shown). At the same concentration of gemcitabine, RT-112 cells were less sensitive compared to MGH-U3 cell line after 3 days of incubation (Figure 6) and that remained the same after 7 days of incubation. Figure 6 demonstrates the minimally affected CRL-1120 after 3 days of gemcitabine exposure.

Clonogenic assay of the cytotoxic effect of gemcitabine on rat TCC

We demonstrated a dramatic dose-dependent cytotoxic effect of gemcitabine on the rat TCC cell line AY-27. After 6 h exposure there was a 1-log kill in the 1×10^{-6} M dosage, and $\sim$2.5-log kill with the 1×10^{-5} and 1×10^{-4} M treatments (data not shown). Since the other cell lines (MGH-U3, RT-112 and CRL-1120) did not yield discrete

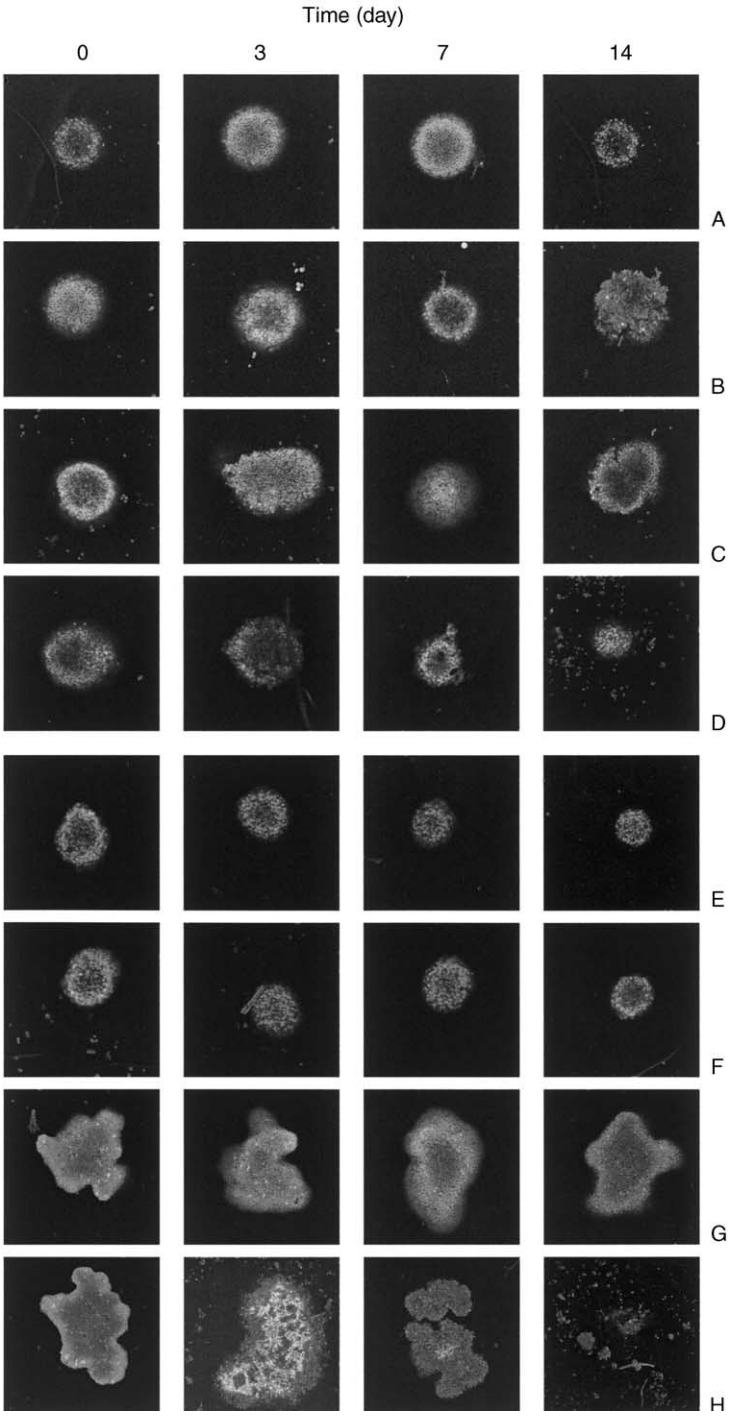

Figure 4. Vital staining for time-dependent selective cytotoxicity of gemcitabine. Vital dye (Syto-16/PI) staining of single (MGH-U3, CRL-1120 and RT-112) and co-cultured spheroids (MGH-U3/CRL-1120), demonstrating the selective cell killing of gemcitabine. Spheroids (single and co-cultured) were treated with gemcitabine for incremental time intervals (0, 3, 7 and 14 days) and confocal images obtained after vital dye staining. Confocal images of: MGH-U3 cells cultured alone as spheroids and treated (B) or not treated (A) with gemcitabine; co-cultured spheroids composed of both MGH-U3 and CRL-1120, treated (D) or not treated (C) with gemcitabine. Confocal images of CRL-1120 fibroblasts cultured alone as spheroids and treated (F) or not treated (E) with gemcitabine, and spheroids composed of RT-112 cells alone and treated (H) or not treated (G) with gemcitabine

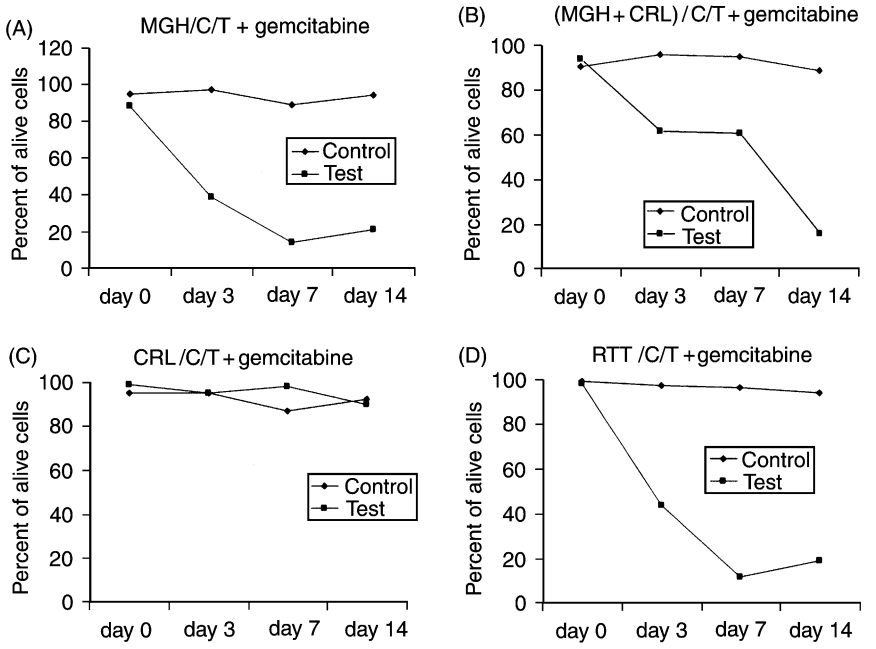

Figure 5. Quantification of cell viability using spheroids confocal images. Images from spheroids made of MGH-U3 alone (A), MGH-U3 with CRL (B), CRL cultured alone (C) and RT-1120 cultured alone (D) were quantified using the Metamorph software program. The majority of cells were dead in spheroids composed of either MGH-U3 or RT-1120 cells alone (A and D). Co-cultured spheroids composed of MGH-U3 and CRL (5B) showed, after 3 days of exposure, the destruction of more than 40% of MGH-U3 cells. The exposure time was proportionally related to the killing of these cells. When the spheroids were made of cultured CRL alone, gemcitabine exposure of 14 days have a non-significant effect on fibroblast cells (C).

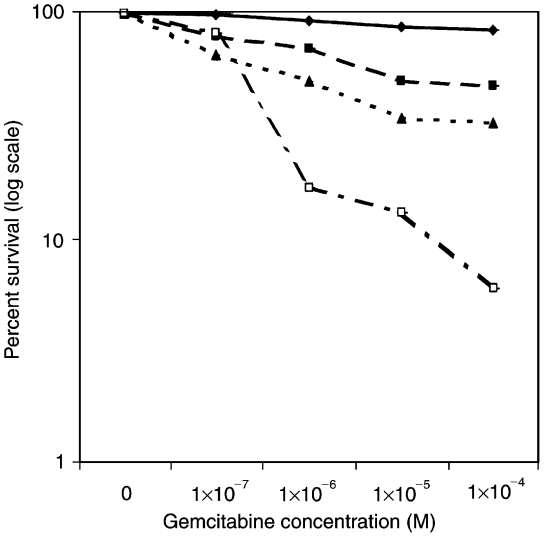

Figure 6. Cytotoxic effect of gemcitabine on TCC and normal fibroblasts using the MTT assay. TCC cells (triangles, MGH-U3; solid squares, RT-112; and open squares, AY-27) and fibroblast cells (diamonds, CRL-1120) display different sensitivity to increasing concentration of gemcitabine.

colonies, we were unable to perform comparative clonogenic assays.

Discussion

Improvement of intravesical therapy requires either improved anticancer efficacy and/or reduced damage to adjacent healthy tissue.[17] Several arguments suggest that intravesical gemcitabine might be a highly effective therapy for superficial bladder cancer. As gemcitabine has substantial activity against advanced urothelial tumors when administered i.v., it is reasonable to expect similar activity in earlier stage bladder carcinoma. Gemcitabine has minimal local toxicity when given s.c.; this implies that normal bladder mucosa might well tolerate gemcitabine exposure. Additionally, gemcitabine appears to be capable of penetrating bladder mucosa[9] and, at high concentrations, entering the systemic circulation. Although bladder penetration raises the potential for systemic toxicity, it may be beneficial in the

treatment of early invasive bladder cancer (T1 disease).

We have established an *in vitro* model consisting of a three-dimensional co-culture of TCC cells and normal fibroblasts for pre-clinical evaluation of new treatment modalities. Since it is difficult to culture normal bladder epithelial cells *in vitro* to serve as control in the design of these three-dimensional co-culture experiments, we opted for normal fibroblasts as a reference because they co-exist well with tumor cells. Despite differences in origin between the mesenchymal fibroblasts and the epithelial TCC, we hypothesized that fibroblasts react to cytotoxic and viral agents in the same way as epithelial cells do. This hypothesis was supported by an *in vivo* study using both spheroids composed of TCC/fibroblasts and a rat orthotopic bladder tumor model.[18] A cytotoxic agent (reovirus) was applied to both models in an independent experimental set-up. Interestingly, normal epithelial cells surrounding the tumor foci in the bladder, as shown during the histology examination of the bladder, profiled the same tolerance as the fibroblast after an exposure to reovirus (Hanel *et al.*, submitted).

Clonogenic assay confirmed that gemcitabine produced cytotoxicity in rat TCC lines at low concentrations, while it had little effect on fibroblast cells as shown by MTT assay. We set out to determine whether gemcitabine exhibited cancer-specific cytotoxicity to transitional cell carcinomas using our spheroid co-culture *in vitro* model. Using immunohistochemistry, co-culture spheroids were demonstrated to polarize with epithelial cells growing peripherally and fibroblast cells centrally. Time-lapse photography was used to monitor the cytotoxic effect of gemcitabine, and it demonstrated that spheroids composed exclusively of MGH-U3 or RT-112 TCC cells were highly gemcitabine sensitive. Spheroids composed of the human CRL-1120 fibroblasts were relatively gemcitabine resistant. In spheroid co-culture models, gemcitabine-treated spheroids have selective loss of the TCC (MGH-U3 and RT-112) components with preservation of the central fibroblast components (CRL-1120). Similar results where obtained with the rat TCC cell line and rat fibroblast co-culture spheroids exposed to gemcitabine. Vital staining of such spheroids demonstrated that gemcitabine had no appreciable cytotoxic effect on the CRL-1120 fibroblast spheroids, whether single or in co-culture with TCC cells, despite achieving highly efficient cell killing of the TCC cells.

To explore the potential protection conferred by the internal location of the CRL-1120 cells in the spheroid co-culture studies, we performed MTT assays on plated cells. Here we found that CRL-1120 was intrinsically less sensitive to gemcitabine cytotoxicity than the MGH, RT-112 and AY-27 cells (Figure 6). This result suggests that such cells were not simply protected from gemcitabine cytotoxicity by the possibility of decreased gemcitabine concentrations achieved in the central portion of our co-cultured spheroids, but rather that the fibroblast cells were intrinsically less gemcitabine sensitive than the malignant cells studied. This was further explored by showing lack of sensitivity of normal fibroblast cells, by using pure CRL-1120 spheroids exposed to gemcitabine.

Live/dead assay of gemcitabine-treated spheroids and non-treated controls showed that more than 70% of the initial number of cells in homogeneous TCC spheroids were dead after 3 days of exposure (Figure 5A and D). At 14 days of gemcitabine exposure, more than 90% destruction of TCC cells was observed.

In the co-cultured spheroids (TCC/fibroblast) (Figures 5B), more than 40% of the TCC cells vanished after 3 days of exposure, whereas 14 days exposure was fatal for at least 95% of the population of TCC cells. Fibroblast composing the same heterogeneous spheroid and subjected to the same conditions of gemcitabine exposure remained intact (Figure 5B). In cultured spheroids composed exclusively of fibroblasts (Figure 5C), most cells survived despite gemcitabine exposure. Fibroblast spheroids tested after 14 days treatment were similar to control in shape, size and dynamic character of living cells.

Conclusion

In aggregate, these results demonstrate that gemcitabine produces selective cytotoxicity to human and rodent TCC cell lines with relative sparing of fibroblast cells when studied in *in vitro* model systems. This observation provides further rationale for performing human clinical trials of intravesical gemcitabine for the treatment of superficial bladder cancer; the observed selectivity of gemcitabine for TCC cells suggests that intravesical exposures sufficient to induce cytotoxicity in superficial bladder cancer cells might produce tolerable levels of normal bladder tissue toxicity. To confirm this hypothesis and further explore the selectivity of intravesical gemcitabine therapy, *in vivo* studies of intravesical gemcitabine therapy are underway in our orthotopic rat bladder tumor model.[18]

Acknowledgments

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