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Gemcitabine: Selective cytotoxicity, induction of inflammation and effects on urothelial function

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ABSTRACT

Intravesical gemcitabine has recently been introduced for the treatment of superficial bladder cancer and has a favourable efficacy and toxicity profile in comparison to mitomycin c (MMC), the most commonly used chemotherapeutic agent. The aim of this study was to assess the cytotoxic potency of gemcitabine in comparison to MMC in urothelial cell lines derived from non-malignant (UROtsa) and malignant (RT4 and T24) tissues to assess selectivity. Cells were treated with gemcitabine or mitomycin C at concentrations up to the clinical doses for 1 or 2 h respectively (clinical duration). Treatment combined with hyperthermia was also examined. Cell viability, ROS formation, urothelial function (ATP, acetylcholine and PGE2 release) and secretion of inflammatory cytokines were assessed. Gemcitabine displayed a high cytotoxic selectivity for the two malignant cell lines (RT4, T24) compared to the non-malignant urothelial cells (UROtsa, proliferative and non-proliferative). In contrast, the cytotoxic effects of MMC were non-selective with equivalent potency in each of the cell lines. The cytotoxic effect of gemcitabine in the malignant cell lines was associated with an elevation in free radical formation and was significantly decreased in the presence of an equilibrative nucleoside transporter inhibitor. Transient changes in urothelial ATP and PGE_2 release were observed, with significant increase in release of interleukin-6, interleukin-8 and interleukin-1β from urothelial cells treated with gemcitabine. The selectivity of gemcitabine for malignant urothelial cells may account for the less frequent adverse urological effects with comparison to other commonly used chemotherapeutic agents.

1. Introduction

At time of diagnosis, 60–80% of bladder cancers are superficial carcinomas, and transurethral resection of the tumour is the first line of treatment followed by intravesical instillation of immunotherapy or cytotoxic drugs (Shelley et al., 2010). Chemotherapy via the intravesical route promotes direct exposure of the tumour to high concentrations of the cytotoxic agent, whilst limiting systemic absorption and consequent systemic side effects. However significant local urological side effects still occur. The most commonly used chemotherapeutic agents are mitomycin C (MMC) and doxorubicin and their use is associated with dysuria and increased frequency and urgency of urination in patients post-treatment (Thrasher and Crawford, 1992; Koya et al., 2006). Gemcitabine, a prodrug, requiring transport into the cell primarily through nucleoside transporters before being converted to its active forms is a newer agent introduced for the treatment of superficial bladder cancer and has a more favourable efficacy and toxicity profile in comparison to MMC. The reported tumour recurrence rate in patients 36-months post-initial intravesical therapy is 28% for gemcitabine compared to 39% for MMC, with a smaller proportion of patients experiencing adverse effects in the gemcitabine group (38.8% compared to 72.2% for MMC) (Addeo et al., 2010).

A number of experimental studies have shown incubation at temperatures between 40 and 44 °C to exhibit a multifactorial tumour cell killing effect, with no damage to normal body tissues including the bladder (Fajardo, 1984; van der Zee, 2002). Chemotherapy with hyperthermia treatment have been shown to act synergistically in reducing cell proliferation in a number of bladder cancer cell lines (van der Heijden et al., 2005). Combined treatment also demonstrated a 59% reduction in recurrence of non-muscle invasive bladder cancer (NMIBC) and an improved bladder preservation profile in patients treated with chemo-hyperthermia compared to chemotherapy alone (Lammers et al., 2011).

During intravesical treatment, the inner lining of the bladder, the urothelium, is the tissue directly in contact with the cytotoxic agents. While the urothelium was previously thought to act solely as a distensible barrier protecting underlying tissues from the contents of urine, recent studies have suggested a role in mediating sensory responses to mechanical and chemical stimuli (Birder, 2011). The urothelium responds to stretch during bladder filling by releasing a number of mediators that influence sensory mechanisms, including ATP, acetylcholine and prostaglandins (Hanna-Mitchell et al., 2007; Tanaka et al., 2011).

Extensive studies have focused on enhancing the toxicity of intravesical chemotherapies, with little regard to the effects of these

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treatments on the normal function of the bladder. Thus, little is known about the mechanisms causing the reported urological side effects following intravesical treatment. We have previously investigated the effects of the commonly used chemotherapeutic agent doxorubicin on urothelial cells and detected the presence of pro-inflammatory cytokines interleukins (IL-8 and IL-1β) and also enhanced stretched-induced release of prostaglandin E_2 24 h after treatment (Kang et al., 2013).

The aim of the present study was to investigate the cytotoxic potency of gemcitabine on cultured human bladder cancer cells with comparison to normal urothelial cells. In addition, the effect of gemcitabine and combined hyperthermia treatment on normal urothelial function was assessed in terms of alterations in urothelial mediator release and secretion of inflammatory cytokines.

2. Materials & methods

2.1. Cell culture

The human urothelial cell lines RT4, T24 (from the European Collection of Cell Cultures) and UROtsa (a gift from Professor Scott Garrett, University of North Dakota) were used in this study. RT4 cells are derived from a transitional cell papilloma of the human urinary bladder and are widely used as a model of superficial bladder cancer. T24 cells are derived from a human urinary bladder transitional cell carcinoma and exhibit characteristics of high grade, invasive tumours. RT4 and T24 cell lines were cultured in McCoys 5A culture media (Invitrogen, Victoria, Australia), supplemented with 10% foetal bovine serum (FBS), L-glutamine (0.5%) and penicillin streptomycin (1%) (Invitrogen, Victoria, Australia).

The UROtsa cell line is an immortalized culture of normal human urothelial basal cells and was routinely cultured in low-glucose Dulbecco's modified Eagle's medium (Sigma-Aldrich, NSW, Australia) supplemented with 5% FBS, 1% glucose and 1% penicillin streptomycin (Invitrogen, Victoria, Australia). For experiments, UROtsa cells were used and grown in serum-supplemented or serum-free media. Incubation of UROtsa cells in serum-free media leaves the cells in a non-proliferative state, while UROtsas grown in serum-supplemented media were used as a model of undifferentiated, proliferative urothelial cells (Rossi et al., 2001). All cell lines were maintained at 37 °C in a 5% CO_2 incubator, passaged when 80–90% confluent. Cell viability was assessed by trypan blue exclusion and only cultures with viability >95% were used for experiments.

2.2. Resazurin viability assay

Reduction of the redox dye resazurin to resorufin was used to measure the viability of cell cultures.(Anoopkumar-Dukie et al., 2005) Cells were seeded at a density of 5×10^3 T24 cells per well, 10×10^3 cells per well for RT4 cells and serum-supplemented UROtsas, and 25×10^3 cells per well for UROtsas grown in unsupplemented media in 96-well microtiter plates for 24 h prior to the addition of gemcitabine concentrations up to the clinical dose (40 mg/mL, 150 mM for gemcitabine; 2 mg/mL, 6 mM for MMC). Also, vehicle treated control cells were included in each experiment. After incubation with gemcitabine at 37 °C or 42 °C for 60 min (gemcitabine) or with MMC at these temperatures for 2 h (clinical durations), cells were washed twice with sterile PBS37°C (37 °C) and maintained in drug-free culture medium for up to 72 h (37 °C). Following incubation for 24–72 h, medium above the cells was removed and replaced with fresh medium containing 44 μM resazurin (Sigma Aldrich). After a 1 h incubation, reduction of resazurin to resorufin was determined by fluorescence (excitation 530 nm; emission 590 nm) using a Modulus multimode microplate reader. Under all conditions tested, the extent of resazurin reduction was directly proportional to viable cell counts. Cell viability was also assessed in the presence of 1 μM nitrobenzylthioinosine (NBMPR) (Tocris, NSW, Australia), an inhibitor of the equilibrative nucleoside transporter.

2.3. Reactive oxygen species production after gemcitabine treatment

The production of reactive oxygen species (ROS) was measured using the 2′,7′-dichlorofluorescin diacetate (DCFH-DA) probe (Sigma-Aldrich, Australia). This assay is based on the intracellular oxidation and consequent de-esterification of the non-fluorescent DCFH-DA to the highly fluorescent 2′, 7′-dichlorofluorescein (DCF). The cell lines were seeded in 96-well microtiter plates as previously described. Cells were treated with gemcitabine at 37 °C for 1 h, before being washed with PBS and incubated for a further 72 h in their respective culture media. Cells were incubated with 10 μM DCFH-DA solution, protected from light for 40 min. The production of ROS was quantified using a Modulus Microplate reader (Ex. 530/Em. 590 nm). DCF fluorescence was normalized to controls using corresponding resazurin reduction data.

2.4. Urothelial mediator release

UROtsa cells cultured in unsupplemented media were used for all urothelial mediator release studies. Twenty-four well plates were seeded at a density of 1.2×10^5 cells per well and incubated in unsupplemented media overnight. Cells were treated with gemcitabine (0–15 mM) diluted in serum-free culture media and incubated at 37 °C or 42 °C for 1 h. Incubation medium was aspirated and cells were washed twice with PBS prior to addition of isotonic solution (130 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM NaHEPES, 0.1% BSA, 5 mM glucose) for 15 min. This solution was replaced with 150 μL isotonic solution and incubated for 10 min, before collection for basal mediator release. For stretch release of urothelial cell mediators, 150 μL hypotonic solution (5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM NaHEPES, 0.1% BSA, 5 mM Glucose) was added to the wells and incubated for 10 min before being collected for analysis. Collection of samples for mediator release was also carried out 24 h following gemcitabine treatment.

2.5. ATP, acetylcholine and PGE_2 assays

ATP was measured using a luciferase-luciferin assay kit (Molecular Probes), acetylcholine using a fluorescence-based Amplex® Red Acetylcholine Assay kit (Molecular Probes) and prostaglandin E2 using a monoclonal antibody based assay (Cayman Chemicals). Assays were performed according to manufacturer's instructions and a Modulus microplate reader (Promega) used to measure luminescence (ATP), absorbance (PGE2, 420 nm) and fluorescence (Ach, Ex.540/ Em.590 nm).

2.6. Inflammatory cytokine analysis

Cell free media was collected from T25 culture flasks 24 h after a 1 h gemcitabine (15 mM) treatment of UROtsa cells. The presence of inflammatory cytokines (IL-1β, IL-6, IL-8, IL-10, 12p70 and tumour necrosis factor) was analysed using a BD Cytometric Bead Array Human Inflammatory Cytokines Kit according to the manufacturer's protocol. Standard and sample fluorescence were analysed on a BD FACS Calibur flow cytometer. The concentration of inflammatory cytokines measured were normalized to controls using corresponding resazurin reduction data.

2.7. Statistical analysis

Results are expressed as mean ± SEM and normalized by cell number where appropriate. Data was analysed using Student *t*-tests or one-way ANOVA with Dunnett's multiple comparisons test, using Graphpad