

INHIBITION OF PURINERGIC P2X₇ RECEPTOR ACTIVITY ATTENUATES APOPTOSIS EVOKED BY RADIATION BYSTANDER EFFECTS IN BLADDER UROTHELIAL AND FIBROBLAST CELLS

Hypothesis / aims of study

Radiation therapy is used in the clinical management of more than 50% of all cancers. In patients with pelvic malignancies, lower urinary tract symptoms (LUTS) are a common and unavoidable consequence of radiation therapy (1) and this condition is known as radiation-induced bladder toxicity (RIBT). RIBT occurs from direct irradiation of cells within the radiation field and also indirectly, where non-irradiated bystander cells respond to signals released by irradiated cells (2). Bystander signalling may therefore be a significant contributor to the development of RIBT and is the focus of the present study. Radiation causes pathological damage to cells of the bladder wall including the urothelium (3) comprising cell swelling and degeneration with some cells undergoing apoptosis.

We hypothesised that '*radiation bystander effects evoke apoptosis in bladder cells modulated by purinergic P2X₇ receptors*' and tested this by addressing the following specific aims:

- (1) to investigate radiation bystander effects on urothelial cell survival and apoptosis;
- (2) to determine whether ATP mimics the bystander effect and if this is mediated through P2X₇ receptor signalling;
- (3) to elucidate the molecular mechanisms underpinning radiation bystander pro-apoptotic signalling in 2D and 3D models.

Study design, materials and methods

Immortalized human urothelial cells (SV-HUC) and primary human bladder fibroblasts (HBF) were cultured in 2D to subconfluence before being irradiated (2Gy) to generate conditioned medium (CM). Non-irradiated SV-HUC were exposed to CM for 1 hour as a model of radiation bystander signalling. Cells were then studied with: flow cytometry to elucidate the percentage of apoptotic cells; RT-PCR and Western blotting to measure relative mRNA and protein expression respectively; and cell survival assays. SV-HUC were studied with patch-clamp electrophysiology to assess ATP-evoked currents. 3D-organotypic rafts of SV-HUC and HBF were developed in the absence and presence of ATP. Cells and rafts processed for immunofluorescence labelling were imaged with confocal microscopy. Data were generated from 3-5 independent experiments, analysed with Student's t-tests and one-way analysis of variance (ANOVA), and presented as mean±S.E.M with P<0.05 considered as significant.

Results

Application of CM to naïve SV-HUC caused a two-fold increase in the number of cells undergoing apoptosis in flow cytometry experiments (N=3, P<0.05). CM also increased caspase-3 activity, demonstrated by upregulated protein expression of cleaved caspase-3 and cleaved Poly (ADP-Ribose) Polymerase-1 (PARP1) (N=5, P<0.05).

Time-dependent analysis (0, 10, 20, 30 and 60 minutes) of CM from irradiated SV-HUC revealed a gradual increase in ATP levels which was significantly higher (N=3, P<0.05) after 20, 30 and 60 minutes compared to a modest increase in CM from non-irradiated cells, thus indicating a potential pathological role of ATP and purinergic signalling in radiation bystander effects.

The dose-dependent decrease in SV-HUC survival by ATP (0.1-5mM) and time-dependent activation of caspase-3 and cell shrinking (N=3) in SV-HUC and/or HBF by ATP (1mM) indicated the ATP-mediated activation of apoptotic signalling in urothelium and bladder fibroblasts. In addition, the presence of cleaved caspase-3 and pronounced increase in (pMLC2-Ser¹⁹) on raft sections further confirmed ATP-evoked morphological changes and cell death.

Application of ATP (1mM) to SV-HUC, held at -60mV, evoked inward currents (-103.5 ± 50.9pA; N=7) indicating the expression of membrane purinergic receptor-operated channels. Live-cell imaging of intracellular Ca²⁺ levels in fluo-4AM loaded SV-HUC by fluorescence microscopy revealed ATP-mediated increases in intracellular Ca²⁺ levels (Ca²⁺-transients). Pre-treatment of cells with suramin (pan-purinergic receptor inhibitor; 100µM) decreased the amplitude of ATP-evoked Ca²⁺-transients, indicating the role of purinergic receptors. Inhibition of P2X₇ receptors with A438079 (P2X₇ receptor inhibitor; 100µM) reduced the ATP-mediated Ca²⁺-transients consistent with P2X₇ receptor-mediated signalling.

In support of these results, in Western blot experiments, pre-treatment with either A438079 or A740003 (P2X₇ receptor inhibitor; 10µM), appeared to mitigate the effects of ATP by normalising ATP-evoked caspase-3 activity and pMLC2-Ser¹⁹ levels in SV-HUC and HBF (N=3). In contrast, dose-dependent treatment with the P2X₇ receptor agonist, Bz-ATP (0.5-1mM) enhanced caspase-3 activity (N=2). Similar experiments in SV-HUC with depleted levels of P2X₇ receptor (50 nM, siRNA) mimicked the effects of inhibitors. Furthermore in the P2X₇-knockdown SV-HUC, the percentage of cells undergoing ATP-evoked apoptosis was significantly reduced (from 37.5% to 26.3%, N=3, P<0.05) as detected by flow cytometry.

Finally, depletion of caspase-3 expression (50nM, siRNA) in SV-HUC normalised the ATP-mediated increase in pMLC2-Ser¹⁹ levels highlighting the significance of caspase-3 activity during apoptosis.

Interpretation of results

Irradiated urothelial cells release ATP into the medium, greater than basal levels which sensitises non-irradiated (bystander) neighbouring cells by eliciting P2X₇ receptor-mediated Ca²⁺-transients, caspase-3 overactivity, cytoskeletal remodelling and directing the cells towards apoptosis.

Concluding message

Urothelial cells respond to factors released from irradiated cells with induction of pro-apoptotic processes. Targeting ATP/P2X₇R signalling may therefore present a promising therapeutic strategy protecting urothelial damage and minimising the symptoms of RIBT.

References

1. Budäus L, Bolla M, Bossi A, Cozzarini C, Crook J, Widmark A, Wiegel T. Functional outcomes and complications following radiation therapy for prostate cancer: a critical analysis of the literature. *Eur Urol.* 2012;61(1):112-27.
2. Prise KM, O'Sullivan JM. Radiation-induced bystander signalling in cancer therapy. *Nat Rev Cancer.* 2009;9(5):351-60.
3. Rajaganapathy BR, Janicki JJ, Levanovich P, Tyagi P, Hafron J, Chancellor MB, Krueger S, Marples B. Intravesical Liposomal Tacrolimus Protects against Radiation Cystitis Induced by 3-Beam Targeted Bladder Radiation. *J Urol.* 2015 Aug;194(2):578-84.

Disclosures

Funding: Medical Research Council grant MR/M012425/1 **Clinical Trial:** No **Subjects:** NONE