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Original Articles

Growth hormone receptor antagonism suppresses tumour regrowth after radiotherapy in an endometrial cancer xenograft model

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ABSTRACT

Human GH expression is associated with poor survival outcomes for endometrial cancer patients, enhanced oncogenicity of endometrial cancer cells and reduced sensitivity to ionising radiation *in vitro*, suggesting that GH is a potential target for anticancer therapy. However, whether GH receptor inhibition sensitises to radiotherapy *in vivo* has not been tested. In the current study, we evaluated whether the GH receptor antagonist, pegvisomant (Pfizer), sensitises to radiotherapy *in vivo* in an endometrial tumour xenograft model. Subcutaneous administration of pegvisomant (20 or 100 mg/kg/day, *s.c.*) reduced serum IGF1 levels by 23% and 68%, respectively, compared to vehicle treated controls. RL95-2 xenografts grown in immunodeficient NIH-III mice were treated with vehicle or pegvisomant (100 mg/kg/day), with or without fractionated gamma radiation (10×2.5 Gy over 5 days). When combined with radiation, pegvisomant significantly increased the median time tumours took to reach 3× the pre-radiation treatment volume (49 days versus 72 days; $p = 0.001$). Immunohistochemistry studies demonstrated that 100 mg/kg pegvisomant every second day was sufficient to abrogate MAP Kinase signalling throughout the tumour. In addition, treatment with pegvisomant increased hypoxic regions in irradiated tumours, as determined by immunohistochemical detection of pimonidazole adducts, and decreased the area of CD31 labelling in unirradiated tumours, suggesting an anti-vascular effect. Pegvisomant did not affect intratumoral staining for HIF1α, VEGF-A, CD11b, or phospho-EGFR. Our results suggest that blockade of the human GH receptor may improve the response of GH and/or IGF1-responsive endometrial tumours to radiation.

Introduction

Radiotherapy is used to treat approximately 50% of all cancer patients, with varying success. Although recent advances in cancer treatment regimens have improved patient prognosis, failure of local control is still a major clinical challenge [1]. Increased expression in tumour cells of autocrine growth factors and receptors, as well as signal transduction cascades involved in tumour cell proliferation/survival, has been demonstrated to promote radioresistance through multiple means [2,3]. For many common cancers, adding novel molecularly targeted agents to radiotherapy may increase cure rates [4,5]. However, currently the EGFR antagonist cetuximab is the only molecularly targeted agent approved as a radiosensitiser. Thus, identification of novel molecularly targeted radiosensitisers addresses an important unmet clinical need [6,7].

Growth hormone (GH) has a wide range of endocrine, autocrine and paracrine effects on growth and metabolism following its secretion from the anterior pituitary and extra-pituitary sites. These can be through direct effects or through secondary stimulation of hepatic insulin-like growth factor 1 (IGF1) secretion. Substantial evidence implicates systemic circulating and extra-pituitary expression of GH and IGF1 in the pathogenesis and progression of cancer [8–14]. In animals and humans with disrupted GH receptor-mediated signal transduction, the incidence of cancer is significantly reduced [11,12,15,16]. In endometrial cancer, human GH (hGH) expression is associated with specific histopathological features including higher International Federation of Gynecology and Obstetrics (FIGO) tumour grade, myometrial invasion, and ovarian metastases, in addition to a worse prognosis for patients [17,18]. In addition, autocrine hGH enhances the oncogenic characteristics of endometrial cancer cells *in vitro* and increases the growth of RL95-2 tumours following stable forced expression [19].

Abbreviations: ATCC, American Type Culture Collection; EGFR, epidermal growth factor receptor; FBS, foetal bovine serum; GH, growth hormone; HIF1α, hypoxia inducible factor-1α; IGF1, insulin-like growth factor 1; VEGF-A, vascular endothelial factor-A.

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Despite extensive *in vitro* studies demonstrating the potential utility of GH receptor antagonism for the purposes of treating cancer, studies investigating antitumour efficacy *in vivo* are limited. Pegvisomant, a clinically available GH receptor antagonist (Pfizer Inc.), is Food and Drug Administration (FDA) approved for the treatment of acromegaly, a debilitating disease characterised by excessive levels of GH, most frequently due to a GH-secreting pituitary adenoma [20]. Pegvisomant is an hGH analogue in which a single mutation in binding site 2 (G120K) prevents complete functional binding to the cell surface GH receptor dimer [21,22], while 6 of 8 amino acid changes introduced into binding site 1 increase receptor affinity [23]. In addition, pegylation of the resulting receptor antagonist increases its pharmacokinetic half-life [21,22]. A small number of xenograft studies have demonstrated antitumour efficacy for pegvisomant as a single agent [24–27]; however, it remains unclear whether pegvisomant can enhance tumour sensitivity to radiation.

Reports indicating that GH may be a radioprotective agent (reviewed in References [10,28]) led us to investigate whether autocrine GH conferred resistance to ionising radiation in breast and endometrial cancer cell lines [29]. We demonstrated that autocrine GH enhances breast and endometrial cancer cell viability, clonogenic survival and DNA repair following treatment with ionising radiation [29]. Conversely, functional inhibition of GH signalling in endometrial cancer cells, using a specific GH receptor antagonist, sensitised cells to ionising radiation-induced cell death and enhanced the induction of DNA damage [29]. Similarly, Wu et al. recently demonstrated that combining recombinant GH with radiation increased clonogenic survival and reduced DNA damage in a colorectal cancer cell line [30], while expression of *GHR* mRNA or protein in rectal cancer predicted response of tumours to preoperative radiotherapy [31].

The aim of the current study was to determine whether inhibition of the GH receptor with pegvisomant sensitises endometrial cancer cells to radiation treatment *in vivo*, using a xenograft model of human endometrial cancer. We used RL95-2 cells as they have previously been demonstrated to express low levels of GH and that antagonism of the GH receptor enhances radiation sensitivity of this cell line [19,32].

Materials and methods

Cell lines and reagents

The human endometrial cancer cell line RL95-2 was obtained from the American Type Culture Collection (ATCC). Cells were cultured at 37 °C, 5% CO_2 in DMEM/F12 (GIBCO) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and Glutamax. Pegvisomant was kindly supplied by Pfizer.

Determination of effective pegvisomant dose

All experiments were carried out under a protocol approved by the University of Auckland Animal Ethics Committee. Female specific pathogen-free NIH-III mice (approximately 22 g) were administered with 20 or 100 mg/kg/day pegvisomant or vehicle (1.36 mg of glycine, 36.0 mg mannitol, 1.04 mg Na_2HPO_4, and 0.36 mg $NaH_2PO_4{\cdot}H_2O$ per 0.5 ml), subcutaneously (*s.c.*) every day for 5 days. Blood was collected 6 h after the final dose of pegvisomant by terminal cardiac puncture under CO_2 anaesthesia.

Xenograft studies

RL95-2 xenografts were established by the *s.c.* injection of 5×10^6 RL95-2 cells suspended in 50 μl serum free DMEM/F12 medium and Matrigel (1:1; BD Biosciences) 1 cm from the tail base on the midline. Mice were allocated to 4 treatment groups when tumours exceeded 100 mm^3 mean volume; the average volume at start of treatment was 173.6 mm^3, with a standard deviation of 55.1 mm^3. There was no significant difference in pretreatment volumes between groups. Mice were treated with vehicle or pegvisomant (*s.c.* 100 mg/kg every day for 7 days, then every second day until study end), with or without fractionated local tumour radiation (10×2.5 Gy over 5 days). The number of animals in each group was as follows: vehicle n = 11; pegvisomant n = 12; radiation n = 11; radiation + pegvisomant n = 11. Pegvisomant administration commenced 2 days prior to the initiation of radiation. Tumours were locally irradiated with an external beam cobalt-60 unit (dose rate 2.55 Gy/min) using a lateral beam with custom-designed lead collimators. Animals were held in restraining boxes without anaesthesia during irradiation. Tumours were measured three times weekly using calipers until they reached three times the pre-radiation treatment volume. Tumour volume was calculated as $\pi (L \times w^2)/6$, where L is the major axis and w is the minor axis.

Once the volume endpoint was reached, blood was collected by terminal cardiac puncture under anaesthesia, 24 h after the final pegvisomant administration. The extent of hypoxia was evaluated by the hypoxia tracer pimonidazole. Mice were dosed *i.p.* with 60 mg/kg of pimonidazole (Hypoxyprobe-1 kit, Hypoxyprobe Inc.) 90 min prior to euthanasia and tumours were 4% paraformaldehyde-fixed for immunohistochemistry.

IGF1 analysis

Serum IGF1 (ng/mL) was quantified by enzyme-linked immunosorbent assay (ELISA) (Mediagnost, Germany) as per the manufacturer's guidelines.

Immunostaining and determination of hypoxia in xenografts

Paraformaldehyde-fixed tumours were paraffin embedded, sectioned (5 μm), mounted on slides, deparaffinised, and rehydrated. Following antigen retrieval in either 0.01 M citrate buffer pH 6 (for Hypoxia inducible factor-1α (HIF1α), vascular endothelial factor-A (VEGF-A), CD11b, phospho-ERK1/2, phospho-epidermal growth factor receptor (EGFR) and pimonidazole antibodies) or 0.5 M Tris buffer pH 10 (for the CD31 antibody) for 1 h, sections were immunostained with antibodies against CD31 (Abcam, ab28364, 1/100), HIF1α (Abcam, ab2185, 1/400), VEGF-A (Abcam, ab183100, 1/500), CD11b (Abcam, ab133357, 1/300), phospho-EGFR (Y1092) (Abcam, ab40815, 1/600); phospho-ERK1/2 (Thr202/Tyr204, Cell Signalling #4370, 1/150) or pimonidazole adducts (Hypoxyprobe, 1/100) and visualised with a Novolink polymer DS 250 Kit (Leica). Anti-HIF1α, CD11b, phospho-ERK1/2, phospho-EGFR and CD31 antibodies all recognise the mouse and human orthologues of the protein. The anti-VEGF-A antibody was human specific.

Immunohistochemistry quantification

Tumours (n = 6 per treatment group) were sectioned and stained by immunocytochemistry as described above. Slides were examined and images were taken using an automated VSlide scanner (Metasystems). For pimonidazole analysis, the percentage of labelled area was quantitated for the entire tumour section under ×10 magnification. For HIF1α, VEGF-A, CD11b and CD31, at least six different fields (×20 magnification) were chosen randomly from each section. Images (TIFF files) were analysed using ImageJ/Fiji software [33]. For HIF1α, VEGF-A and CD31, thresholds were determined using at least three different images. The determined threshold was then used to analyse all images from sections that were stained in the same staining session (taken at ×20). The background level was calculated from control sections and subtracted for image analysis. CD11b was quantitated using point scoring under ×20 magnification. All immunohistochemistry quantification was performed blinded to treatment group and outcome.

Statistical analysis

Statistical significance was determined by one-way analysis of variance (ANOVA), ANOVA on ranks with Tukey's all-pairwise comparison, or paired *t* tests (within-group comparisons; pre- versus post-treatment). Differences in tumour growth delay studies were assessed using a log-rank test with Holm-Sidak multiple comparison analysis. Statistical analyses were performed using SigmaPlot version 12.5 (Systat Software Inc.). $p < 0.05$ was considered significant.

Results

To determine whether pegvisomant can prevent IGF1 production in NIH-III mice, animals (n = 6) were treated with 5 daily *s.c.* doses of pegvisomant. Treatment with 20 mg/kg and 100 mg/kg pegvisomant reduced serum IGF1 concentrations by 23.0% (58 ± 29 (standard error of the mean, SEM) versus 453 ± 17 ng/ml, $p < 0.05$, $p < 0.001$) and 67.7% (versus 190 ± 10 ng/ml, $p < 0.001$), respectively, when compared with vehicle-treated controls (Fig. 1A). No significant bodyweight loss was observed following pegvisomant treatment at either dose level (Fig. 1B).

Next, to determine if pegvisomant can prevent tumour growth alone or in combination with ionising radiation, NIH-III mice were inoculated with 5×10^6 RL95-2 cells in Matrigel and treated with pegvisomant *s.c.* 100 mg/kg/day for 7 days followed by every second