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Targeting the endoplasmic reticulum mediates radiation sensitivity in colorectal cancer

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ABSTRACT

Background: Radiotherapy is an established treatment modality for early and locally advanced rectal cancer as part of short course radiotherapy and long course chemoradiotherapy. The unfolded protein response (UPR) is a cellular stress response pathway often activated in human solid tumours which has been implicated in resistance to both chemotherapy and radiotherapy. This research has investigated whether the UPR pathway is upregulated in ex-vivo samples of human colorectal cancer and characterised the interaction between radiotherapy and UPR activation in two human colorectal cancer cell lines in vitro.
Methods: In vitro UPR expression was determined in response to clinical doses of radiotherapy in both the human colorectal adenocarcinoma (HT-29) cell line and a radio-resistant clone (HT-29R) using western blotting and quantitative polymerase chain reaction. The UPR was induced using a glucose deprivation culture technique before irradiation and radiosensitivity assessed using a clonogenic assay. Ex-vivo human colorectal cancer tissue was immuno-histochemically analysed for expression of the UPR marker glucose regulated protein 78 (GRP-78).
Results: The UPR was strongly up regulated in ex-vivo human colorectal tumours with 36 of 50 (72.0%) specimens demonstrating moderate to strong staining for the classic UPR marker GRP-78. In vitro, therapeutic doses of radiotherapy did not induce UPR activation in either radiosensitive or radioresistant cell lines. UPR induction caused significant radiosensitisation of the radioresistant cell line (HT-29R $SF_{2Gy} = 0.90$ S.E.M. +/−0.08; HT-$29R_{LG}$ $SF_{2Gy} = 0.69$ S.E.M. +/−0.050).
Conclusion: This suggests that UPR induction agents may be potentially useful response modifying agents in patients undergoing therapy for colorectal cancer.

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1. Background

Bowel cancer is the second most common cancer in the UK (ONS, 2011) with 41,581 new cases in 2011. Neoadjuvant radiation therapy, either as part of short course radiotherapy or long course chemoradiotherapy for rectal tumours is now an established part of pre-operative treatment and advised in the post-operative adjuvant setting if a clear margin was not achieved (NICE, 2011). It has significantly reduced local recurrence rates versus surgery alone (OR 0.49; 95% CI 0.38–0.62) (Giunta et al., 2000). Despite these successes colorectal adenocarcinomas have been characterised amongst the most radiation resistant of solid tumours (Fertil et al., 1984).

In order to meet increasing metabolic demand for oxygen and glucose (Warburg, 1956), solid tumour cells produce a complex range of vascular mediators to induce angiogenesis (Hanahan and Weinberg, 2011). These new vessels have major functional and structural inadequacies, limiting tumour perfusion and resulting in a persistently hypoxic, acidotic and hypoglycaemic tumour microenvironment (Koumenis and Wouters, 2006).

These microenvironment conditions increase the radioresistance of tumours through the activation of cytoprotective pathways to combat the increase in hypoxia-induced radical oxygen species (Dendy and Wardman, 2006; Trachootham et al., 2009), which abrogate cellular damage and inhibit apoptosis. In response to these conditions, hypoxia induced transcription factors are produced, which mediate downstream survival pathways, such as the endoplasmic reticulum stress response (Koumenis and Wouters, 2006).

Protein production requires tightly regulated folding and processing of nascent amino-acid sequences within the endoplasmic reticulum, which relies on appropriate substrate availability such as glucose, oxygen and calcium. Inadequate levels of substrate prevent the correct folding of nascent amino-acid sequences resulting in the build-up of potentially cytotoxic, malfolded, proteins within the luminal domain of the rough endoplasmic reticulum (Ma and Hendershot, 2004) which activates one such stress response pathway: the unfolded protein response (UPR) pathway. Activation of this pathway inhibits further new protein synthesis in the endoplasmic reticulum, enhances

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degradation of malfolded proteins by activation of endoplasmic reticulum associated (protein) degradation (ERAD) and inhibits cellular proliferation to minimise the impact of nutrient and oxygen deprivation on the cell. Ultimately it may result in cellular apoptosis if the stress is prolonged or severe (Ma et al., 2002).

Ionising radiation may induce endoplasmic reticulum stress and up-regulation of the heat shock protein family of molecular folding chaperones, which have been implicated in radioresistant rectal tumours (Allal et al., 2004). Indeed, inhibition of the UPR has been shown to increase radioresistance in caspase-3/7 deficient MCF-7 breast cancer cell lines (Woon Kim et al., 2010), suggesting the UPR plays a pivotal role in driving tumour cell survival. As the UPR is not activated within normal tissues unless subjected to microenvironmental stress, targeting this response may provide a novel strategy of selectively radio-sensitising the cancer cell. This has been demonstrated in oesophageal cancer in vitro (Pang et al., 2013).

This study aimed to examine whether the UPR is activated in ex-vivo human rectal cancer specimens and to model the potential consequences of UPR activation in-vitro.

2. Methods

2.1. Ex-vivo study of UPR activation in human rectal cancers

2.1.1. Sample recruitment

Ethical approval was granted by the South Yorkshire Research Ethics Committee (REC number 11/H1310/4). Patients with colorectal cancer were recruited from follow-up clinics and written informed consent was obtained for retrospective access to their formalin fixed, paraffin embedded, colorectal cancer tissue specimens. Tissue Microarrays (TMA's) were produced from these samples to provide a high throughput and sensitive means of undertaking comparable immunohistochemistry (Simon et al., 2004). Each pathological specimen was sampled 4 times and incorporated within the TMA. Large sections were also analysed to compare tumour and adjacent normal tissue levels of UPR activation.

2.1.2. Immunohistochemistry

Blocks of formalin-fixed paraffin embedded large section tumour and microarrays were sectioned at 4 μm and mounted onto charged glass slides (ThermoScientific, USA). Endogenous peroxidase activity was suppressed by incubating samples with hydrogen peroxide. All sections (including microarrays) were stained using an indirect streptavidin–biotin immunoperoxidase method (Vector Laboratories, USA) for GRP-78 (mouse monoclonal, 1:400, BD Biosciences USA). The substrate chromogen, 3,3′-diaminobenzidine was used to visualise sites of primary antibody binding.

The GRP-78 antibody was validated for immunohistochemistry using a sample of formalin-fixed paraffin embedded glucose deprived HT-29 cells and a negative human tissue control.

Prior to scoring, microarray cores were confirmed to contain tumour by an experienced histopathologist. The stained TMA slide was scored using a semi-quantitative intensity scoring system on a scale of 0 (no stain) to 3 (high intensity staining). Two blinded investigators each independently scored the TMA's following a standardisation exercise and intra-class correlation coefficients of agreement were calculated (ICC (3,k); alpha method). The median intensity score for each specimen was calculated per scorer and added together to create a total score out of 6 (Mcdonald and Pilgram, 1999). Negative controls were run without the primary antibody so as to monitor background staining.

2.1.3. Statistical analysis

Statistical analyses were performed in GraphPad 6 (Prism, USA), SDS (Applied Biosystems, USA) SigmaPlot (Systat, UK) and SPSS 21 (IBM, USA). All average values are presented with their corresponding standard errors or confidence intervals. Experiments were repeated in triplicate or quadruplicate.

2.2. In-vitro study of the effect of radiotherapy on UPR activation

2.2.1. Cell culture and maintenance

The human colorectal adenocarcinoma cell line HT-29 (ATCC, 2004) was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, SUI), supplemented with 1% (v/v) L-Glutamine (Lonza, SUI), 1% non-essential amino acids (Lonza, SUI) and 10% (v/v) heat-inactivated foetal bovine serum (FBS) (Lonza, SUI). All lines were maintained at 37 °C in an incubator with 5% CO_2 (Sanyo, JPN). Cultures were tested every 3 months for mycoplasma and found to be mycoplasma-free.

For assays requiring glucose deprivation, glucose-free DMEM was supplemented with glucose-containing DMEM medium and dialysed FBS to achieve the desired concentration of glucose.

2.2.2. Establishing a radioresistant cell line, HT-29R

A radiation resistant clone of the HT-29 colorectal cancer cell line was created (HT-29R) using a standard serial irradiation technique (Wei et al., 2008). Cells were exposed to 2 Gy fractions of X-ray radiation every 7 days, up to a cumulative dose of 22 Gy. Radiation was delivered using an X-ray generator with a tube potential of 200 kV and current 12.8 mA (Ago X-ray, UK). Cultures were passaged weekly at 3 days post-irradiation using trypsin-EDTA (Lonza, SUI).

Radiation resistance was confirmed using a comparative clonal assay after radiotherapy exposure when compared to the parent cell line at 12 weeks following commencement of radiation exposure. Experiments were performed at uniform 24 hour time points from the point of irradiation to 96 h post-radiation. Control lines were treated identically and were of the same passage number.

Clonogenic survival assays of HT-29 and HT-29R were carried out following administration of a range of irradiation doses, 12 weeks after the initiation of radiation therapy to the HT-29R cell line. The HT-29R resistant line was highly resistant compared to the HT-29 parental line (HT-29P) (Fig. 1), exhibiting a 1.5 fold increase in the surviving fraction (SF) at 2 Gy (HT-29R $SF_{2Gy} = 0.90$ S.E.M. +/−0.08; HT-29 $SF_{2Gy} = 0.60$ S.E.M. +/−0.04). This validated the HT29R line as radiation resistant.

2.2.3. Western blotting

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer at 3 °C for 30 min, mechanically disrupted and centrifuged at 13,200 rpm for 10 min at 3 °C. Protein concentrations were determined by the Bicinchoninic Acid assay (Smith et al., 1985) (Sigma-Aldrich, UK).

Clonogenic Assay of Parental (HT-29 P) versus Derived Radiation Resistant HT-29 line(HT-29 R)

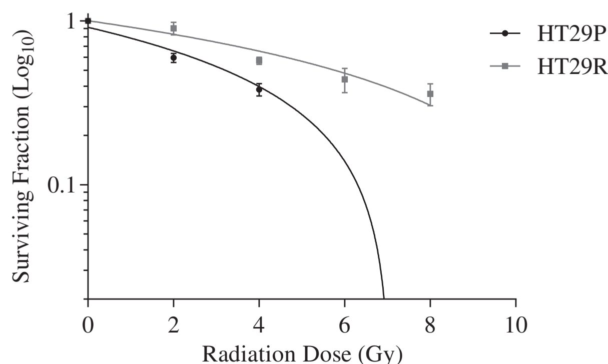

Fig. 1. Survival curves obtained for HT-29 and HT-29R lines. The HT-29R line exhibits radiation resistance across all doses (N = 3) compared to the wild-type HT-29 line. Mean survival with S.E.M.

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