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Radiation quality-dependent bystander effects elicited by targeted radionuclides

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Abstract

The efficacy of radiotherapy may be partly dependent on indirect effects, which can sterilise malignant cells that are not directly irradiated. However, little is known of the influence of these effects in targeted radionuclide treatment of cancer. We determined bystander responses generated by the uptake of radioiodinated iododeoxyuridine ([*I]IUdR) and radiohaloanalogues of *meta*-iodobenzylguanidine ([*I]MIBG) by noradrenaline transporter (NAT) gene-transfected tumour cells. NAT specifically accumulates MIBG. Multicellular spheroids that consisted of 5% of NAT-expressing cells, capable of the active uptake of radiopharmaceutical, were sterilised by treatment with 20 kBq mL^{-1} of the α-emitter *meta*-[^{211}At]astatobenzylguanidine ([^{211}At]MABG). Similarly, in nude mice, retardation of the growth of tumour xenografts containing 5% NAT-positivity was observed after treatment with [^{131}I]MIBG. To determine the effect of subcellular localisation of radiolabelled drugs, we compared the bystander effects resulting from the intracellular concentration of [^{131}I]MIBG and [^{131}I]IUdR (low linear energy transfer (LET) β-emitters) as well as [^{123}I]MIBG and [^{123}I]IUdR (high LET Auger electron emitters). [*I]IUdR is incorporated in DNA whereas [*I]MIBG accumulates in extranuclear sites. Cells exposed to media from [^{131}I]MIBG- or [^{131}I]IUdR-treated cells demonstrated a dose–response relationship with respect to clonogenic cell death. In contrast, cells receiving media from cultures treated with [^{123}I]MIBG or [^{123}I]IUdR exhibited dose-dependent toxicity at low dose but elimination of cytotoxicity with increasing radiation dose (i.e. U-shaped survival curves). Therefore radionuclides emitting high LET radiation may elicit toxic or protective effects on neighbouring untargeted cells at low and high dose respectively. It is concluded that radiopharmaceutical-induced bystander effects may depend on LET of the decay particles but are independent of site of intracellular concentration of radionuclide.

Introduction

After treatment with ionising radiation, cells discharge signals whose effects on neighbouring, unirradiated (bystander) cells include mutation, chromosomal breakage, long-term genomic instability and death (Mothersill & Seymour 2001, 2004; Lyng et al 2002; Little 2003; Lorimore & Wright 2003; Morgan 2003). Bystander effects may be induced by radiation dosage as low as 5 mGy (Mothersill & Seymour 2002). Such exposures are environmentally relevant and also of concern in diagnostic imaging and intensity-modulated radiotherapy. Accordingly, it is important to understand the mechanisms underlying bystander effects and how these may be manipulated to enhance tumour cell kill. Several investigations have demonstrated that radiation-induced biological bystander effects (RIBBEs) may be potent contributors to the efficacy of radiation treatment by eradicating tumour cells that are not directly irradiated (reviewed by Prise et al 2005). Furthermore, bystander effects may also elicit unwelcome consequences in healthy tissue. If bystander responses are involved in carcinogenesis at low doses, the consequences could be substantial, given that the frequency of secondary tumours is rising with improved cure rates by radiotherapy of primary tumour (Brenner et al 2000; Hall & Wuu 2003).

Targeted radionuclide treatment of cancer has developed as a novel and advantageous approach to radiation therapy. The basic idea is to deliver higher doses of radiation to tumour than to normal cells by means of radionuclides chemically conjugated to tumour-seeking targeting agents such as monoclonal antibodies, thymidine analogues or meta-iodobenzylguanidine (MIBG). By virtue of its structural similarity to noradrenaline (Wieland

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et al 1980), MIBG is selectively accumulated via the noradrenaline transporter (NAT) (Jacques et al 1984), which is expressed on the surfaces of cells comprising tumours of neural crest origin (e.g. neuroblastoma and phaeochromocytoma). Radiolabelled forms of this drug are used for scintigraphic assessment and treatment of such tumours (Klingebiel et al 1998; Simpson & Gaze 1998; Hoefnagel 1999; Rose et al 2003). NAT expression is predictive for MIBG uptake capacity (Mairs et al 1994) and quantification of NAT mRNA could be used for the selection of patients for MIBG therapy (Carlin et al 2003).

A limitation to the effectiveness of targeted radionuclide therapy is heterogeneity of uptake of radiopharmaceutical, resulting in underdosing of some tumour regions. This problem could be overcome by the selection of radionuclides whose decay particles have long path lengths, enabling cross-fire irradiation of surrounding untargeted cells. However, even with long-range radionuclides, because their emissions are of low linear energy transfer (LET), sub-populations of tumour cells will receive less than a sterilising dose. On the other hand, emerging evidence suggests that radioactive emission from targeted cells is not the only untargeted effect operating in radionuclide treatment of cancer. It is becoming clear that RIBBEs deriving from the cellular processing of the physical radiation insult, which need not interact directly with DNA, may play an important part in the overall efficacy of targeted radiotherapy.

Understanding the nature of RIBBEs should enable the refinement of radiotherapy. Most investigations of bystander effects have involved external beam γ-irradiators and microbeams. However, recent studies have been conducted of RIBBE after intracellular concentration of radiolabelled drugs. Bishayee et al (2001) prepared clusters composed of unlabelled and [^{3}H]thymidine-labelled cells. The resulting death of unlabelled cells was considered to be a consequence of transfer of toxic factors from cells that had incorporated [^{3}H]thymidine in their DNA rather than cross-fire irradiation because ^{3}H beta-decay particles have a path length that is too short to allow direct bombardment of regions adjacent to targeted cells. Indeed, the mean ^{3}H beta-particle range in tissue is 1 μm (Bishayee et al 2001). Survival of unlabelled cells was increased by treatment with dimethyl sulfoxide and lindane, suggesting the involvement of free radicals and gap junctional communication, respectively. In a similar study, Xue et al (2002) mixed colon carcinoma cells, unlabelled or incubated with the thymidine analogue [^{125}I]-labelled iododeoxyuridine ([^{125}I]IUdR), and used these mixtures to form subcutaneous tumours in athymic mice. Again, inhibition of tumour growth was attributed to RIBBEs because the range of ^{125}I Auger electrons is insufficient to interact directly with neighbouring cells. The latter study demonstrated that RIBBEs are not simply artefacts of in-vitro manipulations but are significant growth inhibitory factors in-vivo (Kassis 2004). It is noteworthy that ^{125}I decays to emit not only Auger electrons but also conversion electrons, which may impinge upon cells nearby. The resulting dose to bystander cells is likely to be much lower than the dose to labelled cells due to Auger electrons (Sastry 1992; Xue et al 2002).

In our own investigations of RIBBEs resulting from cellular concentration of radionuclides, we have utilised cellular monolayers, multicellular tumour spheroids (Boyd et al 2002, 2006) and xenografts (Mairs et al 2007) composed of two populations of cells, only one of which has the ability to transport a radiolabelled drug across the cell membrane. More recently we have made use of media transfer methodology to allow comparison of the direct cytotoxicity of radionuclide uptake with the bystander effect resulting from intracellular accumulation of radiopharmaceutical. Here we describe bystander responses following the uptake of radioiodinated IUdR and radiohaloanalogues of MIBG by NAT gene-transfected tumour cell lines. Our results provide further insight into the dependence of targeted radiotherapy on RIBBE and the influence of radiation quality.

Transfectant mosaic spheroid model

The transfer of therapeutic genes to tumour cells can result in their demise. However, employing current delivery vectors, it is improbable that gene therapy will realise cure because gene transfection is highly inefficient. Consequently, it is obligatory to incorporate into this therapeutic strategy, a constituent that imparts a 'bystander effect' resulting in the death of nearby cells that are not expressing a therapeutic transgene. Hence, to determine the potency of new cancer treatment ploys involving gene transfer, these must be evaluated for their ability to sterilise bystander cells. Commonly, this entails the culture of monolayers composed of a range of proportions of transfected to non-transfected cells and exposure of these two-dimensional models to toxic agents or their inactive precursors (Mesnil & Yamasaki 1999). Such systems have been useful for assessing the requirement for intact gap junctional communication for effective kill (Denning & Pitts 1997) and for the evaluation of strategies to circumvent this requirement (Bridgewater et al 1997). For pharmaceutical-based approaches, the bystander effect relies on the diffusion of activated toxin to neighbouring cells whereas the radiological bystander (or radiation cross-fire) effect inherent in targeted radiotherapy is not mediated by diffusion processes nor is it dependent upon gap junctions.

To assess the impact of treatment on malignant foci in-vitro, a three-dimensional culture system is required. Multicellular tumour spheroids were first described by Sutherland et al (1970). They have been utilised for a variety of experimental applications, including tumour response to various treatments (Mueller-Klieser 1987; Knuechel & Sutherland 1990; Carlsson & Nederman 1992), basic cell biology (Bracke et al 1992; Neeman et al 1997) and investigations of microenvironmental influences on cell growth (Acker & Carlsson 1992; Mueller-Klieser 1997). At diameters greater than 300 μm, spheroids from most cell lines comprise a necrotic core composed of quiescent or moribund cells surrounded by a viable rim of proliferating cells. This structure is similar to the initial avascular stages of solid tumour growth in-vivo, with highly proliferative activity in proximity to capillaries ranging to necrotic regions at greater distances (Sutherland 1988). As a result, multicellular tumour spheroids have been utilised in a variety of experimental cancer therapy studies relevant to the treatment of micrometastases (Knuechel & Sutherland 1990; Gaze et al 1992; Essand et al 1995; Mairs & Wheldon 1996; Mueller-Klieser 1997; Hjelstuen 1998).

Boyd et al (2002) described a modified spheroid model — transfectant mosaic spheroids (TMS) — composed of a range of proportions of transfected and non-transfected cells. These are representative of small tumours in which different efficiencies of gene transfection have been achieved. To discriminate between the different cells, the GFP gene was utilised as a transfection marker for identification, by fluorescence-activated cell sorting (FACS) analysis and fluorescent confocal microscopy (Lybarger et al 1996), of the cells that had not been transfected with the therapeutic transgene — the noradrenaline transporter (NAT) gene. Fluorescence imaging revealed uniform distribution of GFP-expressing transfectants in TMS. The observed heterogeneous dispersal of the two different transfected cell types was independent of their proportional contribution to the total spheroid mass. FACS analysis of disaggregated TMS confirmed that the ratio of NAT-transfectants to GFP-transfectants in various single-cell mixtures used for the production of TMS was accurately reflected in the percentage of the two types of cell in the spheroids (Boyd & Mairs 2006). The TMS model can represent different levels of gene transfer in metastases by a vector that encounters no barrier to its penetration and no impediment to expression of the transgene. However, it should also be possible to create mosaic spheroids composed of transfected peripheral rims or central regions. Such models could be used to represent transfections that are dependent upon, for example, cellular proliferation or hypoxic promotion of expression, respectively.

We have applied our model to the preliminary study of a gene therapy/targeted radiotherapy strategy that confers the potential for uptake of radiolabelled MIBG by introduction of the noradrenaline transporter gene to cells that previously did not express it (Boyd et al 1999). An attractive feature of this approach is the bystander effect in the form of cross-fire irradiation by β-particles from radioconjugates such as ^{131}I. Therefore, not only cells that have been induced by gene transfer to actively accumulate radiopharmaceutical but also neighbouring non-transfectants will absorb a dose of β-radiation. It has been shown that the effectiveness of cell kill by [^{131}I]-labelled compounds is significantly underestimated using monolayer cultures of target tumour cells (Cunningham et al 1998) whereas the impressive collateral damage inflicted by this means is amply demonstrated using three-dimensional cellular aggregates (Neshasteh-Riz et al 1998; Boyd et al 2002).

We used TMS to evaluate the minimum percentage of transfection required to achieve cure of different sizes of metastases. For example: modest radioactivity concentrations (20 kBq mL^{-1}) of [^{211}At]MABG induced the complete sterilisation of all clonogens in 250-μm diameter mosaic spheroids composed of only 5% NAT gene transfectants (Figure 1). Because the path length of ^{211}At α-particles is only 55–70 μm, cross-fire irradiation from targeted to untargeted cells would be considerably less extensive than that from a β-emitter such as ^{131}I. Therefore this observation suggests that bystander effects, over and above cross-fire irradiation, are operating in α-particle targeted therapy. Others have documented bystander effects after α-particle irradiation in situations other than those induced by targeted radionuclides (Nagasawa & Little 1992; Azzam et al 2000, 2001; Hall & Hei 2003; Mitchell et al 2004; Belyakov et al 2005).

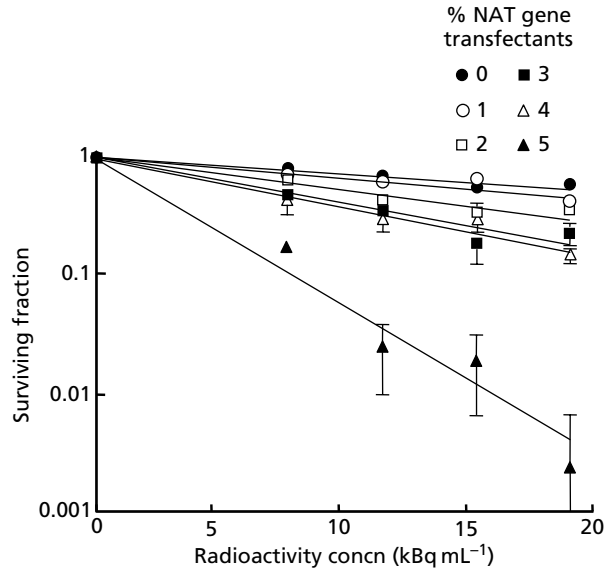

Figure 1 Toxicity, measured by clonogenic assay, of [^{211}At]MABG to UVW spheroids containing various percentages of NAT gene-transfected cells. Means and standard deviations of triplicate determinations.

These studies indicate the potential for bystander-mediated cell kill and improved clinical efficacy of tumour targeting when only a small proportion of the tumour mass expresses the radiotherapeutic molecular target, in this case, introduced via gene modification. Moreover, RIBBEs could compensate for the low levels of gene delivery currently achievable in-vivo in cancer gene therapy strategies when married with targeted radionuclide therapy. Rational selection of radiohaloconjugates of MIBG will enable the enhancement of cross-fire to maximise neuroblastoma cell kill.

Transfectant mosaic xenograft model

To determine the potency of bystander effects in-vivo elicited by cells that accumulated Auger electron emitting radionuclides, Xue et al (2002) incorporated [^{125}I]IUdR into adenocarcinoma cells and mixed these with unlabelled cells before implantation in rodents. They reported inhibition of tumour growth as a consequence of factors released from the labelled cells. The transfectant mosaic xenograft (TMX) model described by Mairs et al (2007) is an alternative means of determining the importance of bystander effects in-vivo. TMX was established by subcutaneous injection into athymic mice of tumour cells comprising various proportions of NAT gene transfected to parental cells — a likely clinical scenario following gene delivery in-vivo.

Xenografts, grown subcutaneously in the flanks of athymic mice and containing no NAT-expressing cell, showed negligible endogenous expression of NAT and negligible uptake of [^{131}I]MIBG. In contrast, NAT gene expression and

[^{131}I]MIBG uptake in TMX, composed of various proportions of NAT-transfected and un-transfected cells, increased in direct proportion to the fraction of NAT-transfected cells.

To assess growth inhibition in this model, representing variably transfected tumours in-vivo, we measured the toxicity of [^{131}I]MIBG to TMX derived from two cell lines and composed of a range of proportions of NAT-expressing cells. In xenografts derived from both cell lines, retardation of tumour growth was observed, even when the tumour contained only 5% NAT-positivity. Moreover, we observed that the inhibition of TMX growth by [^{131}I]MIBG treatment was directly related to the proportion of NAT-expressing cells. In the absence of [^{131}I]MIBG treatment, the level of NAT expression had no effect on the growth rate of xenografts.

Therefore, both NAT gene expression levels and [^{131}I]MIBG uptake accurately reflected the percentage of NAT-positive cells in the tumours in-vivo. This indicates the suitability of TMX for the examination of quantitative aspects of radionuclide treatment and gene therapy. For example, this model will allow, for the first time, the determination of the minimal NAT expression necessary for the imaging and sterilisation by radiolabelled MIBG of tumours of a range of sizes that are one to two orders of magnitude greater than TMS. The TMX model will also facilitate the determination of optimal therapeutic dosage in relation to normal tissue toxicity and the evaluation of the influence of bystander effects in targeted radiotherapy in-vivo.

Media transfer

For determining biological bystander responses in-vitro we employed an adaptation of the media transfer system developed by Mothersill & Seymour (1997). This allowed us to compare the induction of bystander effects by external beam γ-radiation with those generated by MIBG labelled with radionuclides emitting ß-particles, α-particles or Auger electrons (Boyd et al 2006). The cells used in these experiments were transfected with the NAT gene to facilitate the active uptake of radiolabelled MIBG. For investigation of RIBBEs following γ-irradiation, donor cells were directly irradiated and 1 h later their medium was transferred to recipient cells that were not directly irradiated. Twenty-four hours later, clonogenic assay was set up to determine cell kill.

The exposure of two human tumour cell lines — UVW glioma and EJ138 bladder carcinoma — to media derived from external beam irradiated cells produced a dose-dependent reduction in survival fraction in the dose range 0–2 Gy, followed by a plateau in clonogenic cell kill at levels greater than 2 Gy (Boyd et al 2006). Likewise, other reports of media transfer experiments, following treatment with γ-rays or soft X-rays, have indicated that the dose–response in bystander cells reached a plateau at low doses (Mothersill & Seymour 1997; Seymour & Mothersill 2000; Belyakov et al 2001). In contrast, no such plateau with respect to clonogenic cell kill was evident in recipients of medium from NAT-expressing cells incubated with [^{131}I]MIBG. Similarly, no plateau was observed after the treatment with tritiated thymidine of cells in a three-dimensional cluster model (Bishayee et al 2001). Like ^{131}I, ^{3}H is also, to a first level of approximation, low LET radiation. The potency of RIBBEs produced by NAT-expressing cells after treatment with Auger electron emitting [^{123}I]MIBG or α-particle emitting [^{211}At]MABG, increased with activity up to levels that resulted in a direct kill of 40% (EJ cells) or 65% (UVW cells) of clonogens. At higher activity concentrations of [^{123}I]MIBG or [^{211}At]MABG, RIBBEs became progressively weaker. This suggests that in the case of high-LET emissions at low doses, irradiated cells produce a factor that is cytotoxic to bystander cells but, as the dose increases, this signal is perturbed in the irradiated cells perhaps due to the activation of repair processes or apoptotic pathways. The RIBBEs elicited by the pre-nadir activity range of these high-LET targeted radionuclides resulted in a magnitude of cell kill similar to that caused by direct irradiation, indicating that bystander effects may be significant contributors to the cytotoxicity of [^{123}I]MIBG and [^{211}At]MABG at low activity concentrations. It will be important to the development of tumour targeting by [^{123}I]-, [^{125}I]- and [^{211}At]-labelled compounds to determine differences in the nature of signalling molecules generated at low (pre-nadir) and high (post-nadir) radioactivity concentrations.

To determine the effect of subcellular localisation of radiolabelled drugs on direct and indirect cell kill, we treated NAT gene-transfected HCT116 cells (derived from a human colorectal carcinoma) with [^{131}I]MIBG, [^{123}I]MIBG, [^{131}I]IUdR or [^{123}I]IUdR. The thymidine analogue IUdR is incorporated into the DNA of proliferating cells whereas in cells without chromaffin granules, MIBG accumulates in extranuclear sites and has no affinity for DNA (Gaze et al 1991). [^{131}I]MIBG and [^{131}I]IUdR both caused a dose-related decrease in the survival of cells that had concentrated the radiopharmaceutical and in recipients of the medium from irradiated cells (Figure 2 A, C). The concentration of [^{123}I]MIBG and [^{123}I]IUdR by HCT116 cells also induced direct dose-responsive toxicity. However, bystander HCT116 cells succumbed in a dose-responsive manner to the transfer of conditioned medium from donor cultures exposed to low radioactivity concentrations of [^{123}I]MIBG and [^{123}I]IUdR but the toxic effect diminished with increasing dose of radiopharmaceutical to donors (Figure 2B, D). In contrast to our findings, Kishikawa et al (2006) reported that ^{123}I-labelled cells that had been mixed with unlabelled cells stimulated the growth of unlabelled cells in-vitro and in-vivo, whereas the bystander effect induced by ^{125}I was growth inhibitory. This is a surprising observation given the similar electron spectra of ^{123}I and ^{125}I. Our observed biphasic response to treatment with [^{123}I]MIBG or [^{123}I]IUdR was similar to the previously reported effect on the survival of clonogens derived from glioma and bladder carcinoma cell lines and treated with medium from cells that had concentrated [^{123}I]MIBG or [^{211}At]MABG (Boyd et al 2006). Notably, there was a striking similarity in the U-shaped survival curves generated by medium transfer from both [^{123}I]MIBG- and [^{123}I]IUdR-treated cultures. These findings not only support previous observations of the toxicity of Auger electron emitters conjugated to compounds that do not covalently bind to DNA (Sisson et al 1991; de Jong et al 1998; DeSombre et al 2000; Boyd et al 2006), but also indicate that the response of bystander cells to internalised Auger electron emitters, characterised by U-shaped survival curves, is independent of subcellular site of concentration. Furthermore, high LET emitters may have opposing effects on the survival of untargeted cells depending on the administered dose.

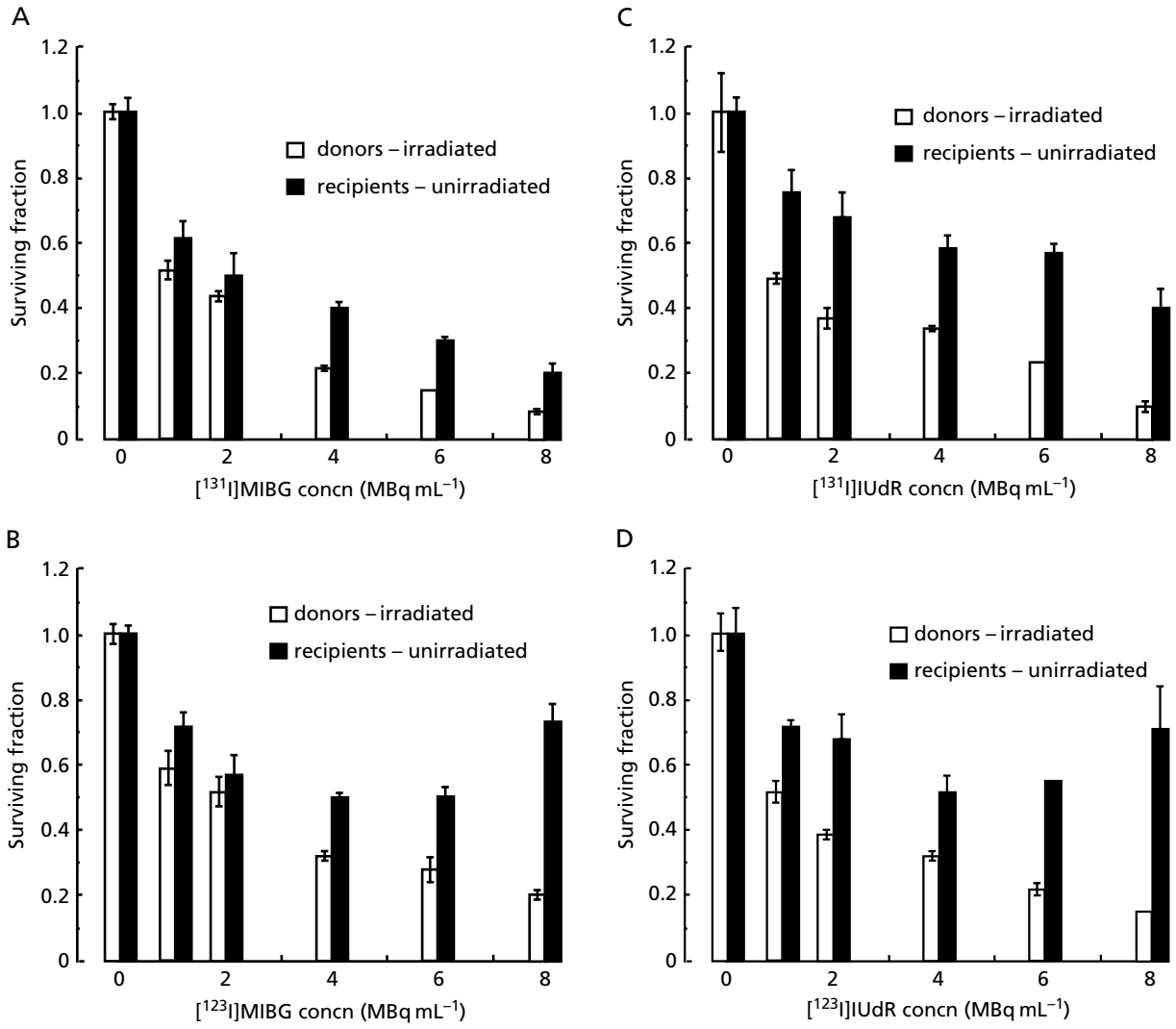

Figure 2 Effect of radiopharmaceutical treatment on clonogenic survival of directly irradiated HCT116 cells and recipients of medium from irradiated HCT cells. Means and standard deviations of triplicate determinations. Surviving fraction is expressed as survival of untreated normalised to unity. A. [^{131}I]MIBG. B. [^{123}I]MIBG. C. [^{131}I]IUdR. D. [^{123}I]IUdR.

Conclusions

The successful application of targeted radionuclide cancer therapy is critically dependent on the delivery of cytotoxic doses of radiation to the vast majority of the malignant cell population. Achieving this goal can be confounded by multiple factors, including variations in target molecule expression and tumour haemodynamic parameters that can lead to heterogeneity in radiopharmaceutical delivery, retention or binding. However, it has been widely appreciated that cells not accumulating the labelled molecule can be killed as a result of being hit by radiation emitted from neighbouring cells. Consideration of this process, known as the (physical) bystander effect, has played an important role in the design of radiotherapeutic strategies (e.g. the selection of long-range β-particle emitters in situations where heterogeneous radiopharmaceutical delivery is anticipated).

A second type of bystander effect that could have important implications for targeted radionuclide therapy is the radiation-induced biological bystander effect (RIBBE), which results in the killing of cells not directly exposed to radiation. The mechanisms involved are as yet undefined. However, studies using γ-ray and α-particle beams have provided some insight into possible factors. These include oxidative stress leading to increased radical formation (Lehnert & Goodwin 1997; Narayanan et al 1997). Oxidative stress is a common feature of several non-DNA-targeted effects of ionising radiation and may be influenced by nitric oxide (Matsumoto et al 2001; Shao et al 2002) and cytokine release (Iyer & Lehnert 2000) as well as factors transmitted via gap-junction intercellular communication (Azzam et al 2001). The RIBBE resultant from targeted radionuclides has largely been unexplored and the effects of radiation quality remain unknown (Mairs & Boyd 2005).

Elucidation of the pathways involved in RIBBE generation by radionuclides could indicate ways of manipulating RIBBE production to reduce toxicity to normal tissues which are inadvertently irradiated during the course of a targeted radiotherapy regime. Careful choice of radionuclide and dose administered in clinical scenarios for targeted radionuclide therapy of tumours that naturally accumulate targeted radionuclides, or have been genetically manipulated to do so, will allow compensation for factors such as inefficient gene transfer and heterogeneous uptake, thereby optimising the cell kill potential of this therapeutic scheme.

Whatever the mechanism, RIBBEs could be important not only in relation to radiation protection and safety but also with respect to the therapeutic use of ionising radiation. Exploitation of RIBBEs could be especially relevant to the efficacy of targeted radiotherapy because this treatment is limited by heterogeneous uptake of radionuclides by tumours. Freely diffusible toxic bystander signals could overcome the inefficiency of tumour control due to non-uniform distribution of radiation dose.

We seek now to investigate the nature of RIBBE signals generated by radiopharmaceuticals localised to different subcellular regions. The efficiency of this mode of kill in tumour and normal cells and its possible dependence on genetic background and tumour microenvironment must also be assessed. From a practical perspective, the identification of RIBBE factors will stimulate the design of strategies to maximise damage to tumour cells while minimising damage to normal cells.

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