Bystander effect

The importance of bystander effects in radiation therapy in melanoma skin-cancer cells and umbilical-cord stromal stem cells

Jaime Gómez-Millán b,1, Iana Suly Santos Katz c,1, Virgínea de Araujo Farias a, Jose-Luis Linares-Fernández a, Jesús López-Peñalver a, Gustavo Ortiz-Ferrón a, Carmen Ruiz-Ruiz a, Francisco Javier Oliver d, José Mariano Ruiz de Almodóvar a,e,*

a Instituto de Biopatología y Medicina Regenerativa, Universidad de Granada, Spain; b Hospital Universitario Virgen de la Victoria, Unidad de Gestión Clínica de Oncología, Málaga, Spain; c Instituto Butantan, Centro de Pesquisa e Formação em Imunologia, São Paulo, Brazil; d Instituto de Parasitología y Biomedicina López-Neyra; and e Hospital Universitario San Cecilio, Granada, Spain

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A B S T R A C T

Purpose: To examine direct and bystander radiation-induced effects in normal umbilical-cord stromal stem cell lines and in human cancer cells.

Materials and methods: The UCSSC lines used in this study were obtained in our laboratory. Two cell lines (UCSSC 35 and UCSSC 37) and two human melanoma skin-cancer cells (A375 and G361) were exposed to ionizing radiation to measure acute radiation-dosage cell-survival curves and radiation-induced bystander cell-death response.

Results: Normal cells, although extremely sensitive to ionizing radiation, were resistant to the bystander effect whilst tumor cells were sensitive to irradiated cell-conditioned media, showing a dose–response relationship that became saturated at relatively low doses. We applied a biophysical model to describe bystander cell-death through the binding of a ligand to the cells. This model allowed us to calculate the maximum cell death ($K_{max}$) in terms of dose equivalence (Gy). The values obtained for $K_{max}$ in A375 and G361 cells were 0.23 and 0.29 Gy, respectively.

Conclusion: Our findings help to understand how anticancer therapy could have an additional decisive effect in that the response of sub-lethally hit tumor cells to damage might be required for therapy to be successful because the survival of cells communicating with irradiated cells is reduced.

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Variation in the intrinsic radiosensitivity of both human-tumor and normal-tissue cells is already recognized, and these differences may be related to clinical curability and tolerance to treatment [1]. At present, choice of the appropriate dose for all patients is based on a balance between minimizing the incidence of severe normal-tissue complications and maximizing the probability of local control. Radiation is an unusual toxic agent in that the timing of tissue damage can vary widely from one patient to the next [2,3]. Radiotherapy is based on traditional radiobiological models, in which the effect of radiation on cells is assumed to result from a cascade of simultaneous or successive events that start with the initial damage to DNA. Radiation-induced lethal or potentially lethal damage to the DNA of cells can be explained by linear-quadratic models, which can also be used to describe the relationship between the total isoeffective dose and dose per fraction in fractionated radiation therapy [4]. Based on these models, successful tumor control requires that all clonogenic cells receive a lethal dose. The initial radiation-induced damage to DNA [5] may be a biological indicator of the quantity of energy transferred to the DNA. Nevertheless, the late effects of radiation have still not been fully explained and thus a more general theory to describe the consequences of radiation therapy appears to be necessary [6]. A key consequence in cells is that direct damage occurs to the DNA within the nucleus, producing a range of lesions, of which DNA double-strand breaks (DSBs) play a vital role in determining whether they survive radiation exposure or not [7]. The presence of DNA damage in cells activates repair mechanisms as well as signal transduction pathways, leading to cell cycle arrest and apoptosis. The tumor suppressor protein p53 plays a key role in whether the cell cycle is arrested or apoptosis ensues after a genotoxic attack. Parp-1 participates in the p53 response following irradiation [8,9], but the cascade of events, including intra- and inter-cellular signaling involving free radicals, reactive oxygen species, cytokines or epigenetic changes, has still to be clarified and the results can vary.
considerably according to the type of radiation and the genotype, DNA repair capacity and physiological state of the cells and tissues involved [10].

Since the bystander effect was first described, the biological meaning and extension of this effect remains open to debate [11]. A great deal of evidence suggests that the application of ionizing radiation to a target volume that includes the tumor elicits effects that exceed radiation-induced cell death and also produces diffusible oxygen reactive species and secreted factors that have been identified as possible mediators in the indirect bystander effect [12], and it can be clearly seen that the damage induced by the bystander effect is not necessarily the same as that responsible for the traditional effects of radiation [13]. The recipient cells transduce the signals produced by irradiated cells and appear to coordinate a response. Such responses recorded to date include the initiation of apoptosis, differentiation and proliferation [14–16]. These coordinated responses can be protective in that an apoptotic response, for example, can remove an abnormal cell from the population, but the response can also involve pre-malignant conditions such as the fixing of mutations, the induction of genomic instability or cell transformation [17]. This knowledge supports a possible change in the way we interpret a patient's response to radiotherapy [15,18] as well as implications for the standard paradigm for radiation risk in cancer sufferers and inheritable effects [19]. As with any other medical procedure, the prescription of a course of radiotherapy must represent a balance between risk and benefit [20,21]. To understand the molecular mechanisms involved in the bystander effect and identify the relationships between molecular interactions and effects we have undertaken experiments designed to examine how the direct and bystander radiation-induced effects for different cell models depend on the cell type and the challenge doses. We exposed cells to ionizing radiation doses of between 0 to 8 Gy to measure the acute radiation dose–cell survival curves for all the cell lines assessed. Potential bystander effects were investigated by medium-transfer experiments [22] using the same doses and the same set of cell lines. With this approach we have tried to throw some light on the contradictory effects mentioned above and to clarify the debate as to whether these can be termed damaging or protective bystander responses, which we hope might lead to possible new strategies to improve the rational use of radiotherapy in cancer treatment.

**Materials and methods**

**Cell lines and culture conditions**

*Mesenchymal stem cells*

It has been demonstrated that human umbilical-cord stromal cells (UCSSCs) are bio-equivalent to bone-marrow mesenchymal stem cells. The UCSSC lines used in this study (UCSSC 35 and UCSSC 37) were obtained in our laboratory and prepared as described elsewhere [23,24] with some modifications. The UCSSCs were cultured as described elsewhere in low-serum culture medium (LSCM) under a 5% oxygen atmosphere, conditions in which their differentiation potentialities are preserved [25]. When a confluence of 70–80% was reached the cells were detached with 0.25% trypsin–EDTA (Sigma) and a suitable number of cells were seeded onto new culture plates to conduct each experiment.

**Human melanoma skin-cancer cells**

The A375 cell line was a gift from Dr. Bosserhoff (Institute of Pathology, Regensburg University, Germany). The cells were cultured in DMEM-LG (Invitrogen, 21885-108) containing 10% fetal bovine serum (PAA), 1% non-essential amino acids (Invitrogen 11140035) and 1% penicillin/streptomycin (Invitrogen, 15140-122). G361 cells were obtained from the cell-culture facility at the University of Granada (CIC) (ref # ECCAC: 8803040) and cultured in DMEM-HG (Invitrogen, 31966-047) containing 10% fetal bovine serum (PAA) serum and 1% penicillin/streptomycin (Invitrogen, 15140-122). The cells were cultured until they reached 70–80% confluence and were then detached using trypsin/EDTA (Invitrogen, 25200-072) and 3 × 10⁵ cells were subcultured in a 25 cm² flask.

**Generation of irradiated cell-conditioned media**

Cells growing exponentially at 70% confluence were irradiated (0–8 Gy) at room temperature at a dose-rate of 2 Gy/min using a 137Cs irradiator. The medium was removed after 24 h, centrifuged at 3000 rpm at room temperature and filtered using 0.22 μm meshes. This medium (RCM) was used for subsequent experiments.

** Colony-forming assay**

Cells were harvested with 0.25% trypsin–EDTA (Sigma) and suspended in full culture medium. Clonogenic assays were performed in 25 cm² flasks (Nunk, Denmark). No feeder cells were required. For clonogenic-cellsurvival assays, A375, G361, UCSSC 35 and UCSSC 37 cells were exposed to 0–8 Gy or to RCM generated from exposure to 0–8 Gy or left untreated. Cells in fresh medium served as controls. The cells were then cultured for 10–14 days and the resulting colonies (>50 cells) were scored. Surviving fractions were calculated as described elsewhere [7].

**Medium-transfer experiments**

Potential bystander effects were investigated by medium-transfer experiments using RCM as described elsewhere [22]. The ability of RCM to induce cell death (χ) in bystander cells was assessed by the different RCM obtained after the irradiation of cell cultures at doses ranging from 0 to 8 Gy. Cell death was measured as χ = 100 – clonogenic cell survival (%). The cultures exposed to RCM were incubated for 24 h, after which this medium was replaced by standard culture medium. Subsequent studies were conducted with RCM exposed to 6 Gy radiation. The cells were then cultured for 14–18 days and the resulting colonies (>50 cells) were scored. Clonogenic cell survival (%) was calculated as described elsewhere [7].

**Analysis of apoptosis**

After 24 h treatment cells with RCM (0–4 Gy), hypodiploid apoptotic A375 and G361 cells were detected by flow cytometry according to published procedures [26]. In brief, the cells were washed with phosphate-buffered saline (PBS) and fixed in 70% cold ethanol. Specific DNA was extracted from apoptotic cells using a phosphate/citric-acid buffer at pH 7.8, treated with RNase and then stained with propidium iodide. Quantitative analysis of the cell cycle and sub-G1 cells was carried out in a FACS CANTO II cytometer using the FACS DIVA software (Becton Dickinson, Mountain View, CA, USA).

**Comet assay**

Single-cell gel electrophoresis (comet assay) was used to calculate DNA damage and repair in melanoma skin-cancer and h cells. At different times after irradiation (6 Gy) floating and adherent cells were collected and 10 µl aliquots of 1 × 10⁵ cell/mL in PBS were embedded in 120 µL of 1% low-melting-point agarose in PBS at 37 °C. The cells were then transferred to microscope slides, protected with a coverslip and left in the refrigerator for 5 min, after which the coverslip was removed and the slides were processed as described elsewhere [27]. The slides were studied at 100x magnification. DNA damage was determined by evaluating 100 cells per sample. Tail moment was measured using the CASP software.
Fractionation in the bystander effect

To investigate the contribution of the bystander effect to a course of fractionated treatment we conducted two different experiments: firstly to measure the ability of RCM to induce cell death in bystander cells by successive dilutions (1/1 to 1/5) of a medium obtained after irradiating tumor cells with 6 Gy, and secondly to ascertain the overall effect of reiterating the treatment on the same tumor-cell population via a medium-transfer experiment in a fractionated regime of 24 h exposure to RCM, changing the medium four times over a period of 4 days. After each change of medium a group of flasks was separated to proceed with the colony-forming assay, assessing the clonogenic cell surviving fraction after 10–14 days' culture. The intention of this experiment was to simulate the potential bystander effect produced by fractionated radiotherapy treatment on a tumor.

Results

Cell survival after irradiation

Models of cell death by radiation are usually presented in the form shown in Fig. 1. Two descriptions of the shape of survival curves can be used with a minimum of mathematics. For very sensitive human cells the survival curve is a straight line from the origin; that is, survival rate is approximately an exponential function of dose. Although some experimental data fit such a model quite satisfactorily, there are also many data sets, in particular those concerning tumor cell lines, that often fit more closely to a curve with a finite initial slope and some form of bending component [28]. Our results show that for UCSSCs the survival curve can be described by Equation [I] and one parameter, the slope ($\alpha$) of the line:

$$S = e^{-\alpha D}; \quad D_0 = \frac{1}{\alpha}$$

where $S$ is the fraction of cells surviving a dose $D$, $\alpha$ is the slope and $D_0$ the reciprocal of the slope that depicts the dose required to deliver one inactivating event per cell on average.

Survival curves for melanoma skin-cancer cells irradiated in vitro are, however, shouldered and the continuous downward-curving form of a cell-survival experiment can by fitted by the linear-quadratic (LQ) model. The formula for cell survival [29,30] is

$$S = e^{-(\alpha D + bD^2)}$$

Survival curves after irradiation are presented here with doses plotted on a linear scale and survival on a logarithmic scale. Both curves, purely exponential for UCSSCs and a linear-quadratic model for melanoma skin-cancer cells, suitably describe cell response to radiation above 1 Gy. The surviving fractions at 2 Gy were 26.9 ± 1.8 and 21.2 ± 2.1 for UCSSCs vs. 64.8 ± 3.2 and 59.7 ± 2.3

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**Fig. 1.** Clonogenic dose–response curves in directly irradiated cells: (A and B) A375 and G361 human melanoma cells and UCSSC 35 and UCSSC 37 normal stem cells (differences between cancer and normal stem cells are significant [paired t test, $P < 0.0001$]). (C and D) kinetics of DNA strand-break rejoining after exposure to 6 Gy of $\gamma$-radiation in melanoma cancer cells and normal human stem cells as measured by comet assay. Mean ± SE values of at least two experiments (each run in duplicate) are given for each cell line. Mean ± SE values of at least two experiments (each run in duplicate) given for each cell line (*$P < 0.05$; **$P < 0.001$).
The rate of reparation of DNA strand breaks

Following damage induction, numerous processes remove and repair the damage in an attempt to restore the genetic sequence to its original state. Fig. 1C and D shows the kinetics of DNA repair for the two cell models studied, measured according to the decrease in tail moment in the comet assay. To evaluate this process quantitatively we used the measure of tail moment after 4 h repair compared to its initial value. This parameter, expressed here as the mean value ± standard deviation, turned out to be 10.6 ± 2.6% and 40.32 ± 6.97% for melanoma skin-cancer cells and 50.67 ± 6.97% and 67.2 ± 9.4% for human umbilical-cord cells (P < 0.001, and P < 0.05, Fig. 1C and D). Thus it is clear that cancer cells repair DNA damage more readily than UCSSCs do.

Medium-transfer experiments

A key characteristic of bystander responses, as opposed to direct irradiation effects, is the dose–response relationship. Instead of an increased response concomitant with an increase in radiation dose, the bystander response becomes saturated at relatively low doses (Fig. 2A). This may suggest a receptor–ligand interaction, which we took as our initial hypothesis, with the characteristic of being both dynamic and reversible. At equilibrium, even though the concentrations of free receptor and ligand and the bound product are constant, free and bound molecules are continually being transformed into one another. According to the law of mass action, the initial concentrations of receptor and ligand determine the equilibrium position.

\[ [R] + [L] \xrightarrow{k_1} [RL] \xrightarrow{k_2} [\chi] \]

As cell death is the end-point analyzed here, in accordance with our hypothesis, [R] is the concentration of death receptor, [L] the concentration of ligand, [RL] the complex formed and [\chi] the product, in this case cell death.

In melanoma skin-cancer cells the rate of cell death [\chi] increases concomitantly with dose (Gy) until a maximum value is reached (Fig. 2A). At this point it should be clarified that the concentration of receptor must be taken as constant for each experimental point because the number of cells in all cases is constant. Nevertheless, the concentration of ligand in the irradiated cell-conditioned medium depends on the dose with which the cells for melanoma cells. For doses of less than 1 Gy, however, our results seem to fit the low-dose hyper-radiosensitivity phenomenon [31]. The clonogenic cell-survival parameters and the survival curves for two melanoma skin-cancer cells and two UCSSCs (A375; G631; UCSSC 35 and UCSSC 37) after irradiation are shown in Table 1 and Fig. 1A and B.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>( \sigma (\text{Gy}^{-1}) )</th>
<th>( \beta (\text{Gy}^{-2}) )</th>
<th>SF(_2) (%)</th>
<th>( D_0 ) (Gy)</th>
<th>Plating eff</th>
</tr>
</thead>
<tbody>
<tr>
<td>G361</td>
<td>0.131 ± 0.023</td>
<td>0.043 ± 0.003</td>
<td>64.8 ± 3.2</td>
<td>3.52</td>
<td>29.3 ± 2.5</td>
</tr>
<tr>
<td>A375</td>
<td>0.187 ± 0.057</td>
<td>0.035 ± 0.008</td>
<td>59.7 ± 2.3</td>
<td>3.15</td>
<td>58.8 ± 3.2</td>
</tr>
<tr>
<td>UCSSC 35</td>
<td>0.657 ± 0.018</td>
<td>0.269 ± 1.8</td>
<td>1.15</td>
<td>14.7 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>UCSSC 37</td>
<td>0.754 ± 0.043</td>
<td>0.221 ± 2.1</td>
<td>1.33</td>
<td>15.0 ± 0.6</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Characteristic values of the cell lines used.

The difference between theoretical values according to the equation [\chi] and experimental values of cell survival after irradiation fitted to the lineal-quadratic model. Maximum differences are indicated along with the corresponding dose values. The differences at 2 Gy are also indicated.

Fig. 2. Medium-transfer experiment producing bystander responses. Melanoma skin-cancer cells are sensitive to RCM (A) whereas UCSSC 35 (■) and UCSSC 37 (■) are not (B). Values for \( \chi_{max} \) and \( K_{B0} \) are summarized in Table 1. (C) Effect of RCM used in successive dilutions on the clonogenic capacity of A375 cells. (D) Reiteration of RCM treatment (4 times at 24-h intervals).
are treated and on the ability of these cells to secrete cell-death ligands. Thus, given that $[R]$ is constant and $[L]$ variable until a maximum is reached, as radiation-induced cell death increases concomitantly with dose, and both phenomena – active secretion and cell death are mutually exclusive – it is reasonable to presume that the process becomes saturated.

The formula for this mathematical model is

$$x = x_{\text{max}} \cdot \frac{D}{K_{\text{By}} + D}$$

This equation describes the binding of a ligand to a receptor following the law of mass action, where $x$ represents cell death, $x_{\text{max}}$ is maximum cell death, $D$ the dose at which the irradiated-cell-conditioned medium (RCM) was obtained and $K_{\text{By}}$ the dose administered to get a RCM for which the cell-death rate is half-maximum. We took this value to be a measure (Gy-equivalent) of how sensitive the cell model with a low $K_{\text{By}}$ value (corresponding to the most sensitive cells) is. The values of $x_{\text{max}}$ and $K_{\text{By}}$ are summarized in Table 1 and by comparing them we can see that A375 cells were slightly more sensitive to the bystander effect than G361 ($K_{\text{By}}$ 0.23 vs. 0.29 Gy) and that stem cells taken from both umbilical cords (UCSSC 35 and UCSSC 37) were insensitive to RCM generated after the treatment of melanoma skin-cancer cells (Fig. 2B).

Fractionation in the bystander effect

The results described above prompted us to undertake two different experiments to study the dose-dependence of the relationship more closely. First we measured the effect of the RCM (6 Gy) used in successive dilutions (Fig. 2C) on the clonogenic capacity of A375 cells. An analysis of this figure revealed that the higher the dilution the lower the bystander effect. Thus we may conclude that in our experiments the percentage of cell death per unit of ligand concentration present in any volume of the RCM added to the non-irradiated cell culture was constant ($k = 19.5\%$; $P < 0.001$). We then assayed the reiteration of RCM treatment (4 times at 24-h intervals) to the same tumor-cell culture flask (Fig. 2D). The data summarized in the figure show that each fraction of treatment produced a progressive decrease in the clonogenic cell survival of non-irradiated A375 cells. Using a purely exponential model of the relationship between treatment and cell survival we can say that fractionated treatment results in a reduction in survival of 24% per fraction of treatment ($P < 0.0001$).

Analysis of apoptosis

After our irradiated medium-transfer experiments we checked to see whether cell death mediated by the interaction between the soluble factors present in RCM and its receptor in the tumor-cell membrane followed the apoptotic pathway. Our results seem to corroborate that the loss of clonogenic capacity in melanoma skin cancer, both in A375 and G361 cells, was due to a typical apoptotic process. This is shown for A375 cells in the flow-cytometry histograms (Fig. 3A) and in the dose–response relationship found between the radiation dose used to produce the RCM and the proportion of cells in the sub-G1 area (Fig. 3B and C). The differences between the control and the 1 and 2 Gy groups are statistically significant ($P < 0.05$).

![Fig. 3. Hypodiploid apoptotic cell detected by flow cytometry after treating the melanoma skin-cancer cells with RCM for 24 h.](image-url)
Discussion

Clinical radiotherapy for cancer inevitably exposes the entire body of the patient to some radiation through the radiation-induced bystander signals. The prime goal of therapy is to destroy tumor cells, but anticancer therapy could have an additional decisive effect: the sub-lethally hit tumor cells' response to damage might be required for the therapy to be successful [31,32] because communication with irradiated cells reduces their survival rate. Over recent years there have been many studies into the bystander effects of radiation and evidence now shows that as well as direct DNA damage-dependent effects, irradiated cells also send signals to their neighbors, causing a wide variety of possible effects in bystander cells. All these effects seem to be substantially more pronounced at low-to-moderate doses, with little or no further increase at higher doses [17,19].

DNA damage and cell survival after radiation

Using the comet assay we found that, compared with normal UCSSCs, melanoma skin-cancer cells exhibited a faster rate of strand-break repair and saturation of DNA damage recovery ( \( \leq 4 \) h) and also showed lower levels of unpaired DSBs later after irradiation (Fig. 1C and D). These differences are reflected in the results found in clonogenic cell-survival experiments (Fig. 1) because in the more sensitive UCSSCs the \( D_0 \) value (the dose that reduces the surviving cell fraction to 37%) ranged from 1.25 to 1.68 Gy, which corresponds with the slower DNA rejoining kinetic (67.2 ± 9.4% and 50.65% of the initial radiation-induced DSBs remained unrepaired 4 h after irradiation), whereas with melanoma skin-cancer cells \( D_0 \) ranged from 3.05 to 3.62 Gy and the quantity of DSBs that remained unrepaired after 4 h was only 10.6 ± 2.6% and 40.32% in each of the melanoma skin-cancer cell lines tested. These results are consistent with previous works [33] and we know that stem cells represent a cell model characterized by their high radiosensitivity values against the direct effect of ionizing radiation [34].

Clonogenic survival in bystander cells

Our results (Fig. 2A) support the hypothesis that bystander effects may be a widespread phenomenon [17] that occurs when tumor cells are in contact with the culture medium in which cells of the same type have been irradiated. Nevertheless, normal cells, represented in our experiments with UCSSCs, although extremely sensitive to ionizing radiation, are resistant to any bystander effect induced with RCM generated after irradiating A375 and G361 cells at doses of between 0 and 8 Gy (Fig. 2B). A lack of evidence for any radiation-induced bystander response in normal human fibroblasts [35], embryonic stem cells [36] and mesenchymal cells [37], even when these cells are transduced with a retroviral vector encoding full-length human TRAIL [38], has been described elsewhere. Nevertheless, a robust bystander response has been observed in cancer cells [39–41].

We have applied here a biophysical model to describe how cells bind ligands and turn them into products, cell death being the conclusion of the catalytic process initiated by the binding of the ligand to the cell surface receptor.

Although our results might not be considered as definite proof of the mechanisms of the bystander effect we can safely say that the mathematical model conforms closely to the experimental values ( \( P < 0.0001 \)). We observed a saturation in the bystander effect in both A375 and G361 cells, with statistically different values for \( \chi_{max} \) and \( K_{by} \) (Table 1) when both dose–response curves (Fig. 2A) were compared.

Cell-death modality

One major drawback with cancer therapy is the development of resistant cells within tumors and therefore the bystander effect may have great implications in cancer radiotherapy as an additional mechanism able to propagate tumor cell death. Experiments undertaken using A375 and G361 cells and flow cytometry to measure DNA fragmentation (fraction of cells in the sub-G1, region of the histogram) show that in melanoma cancer cells after treatment with RCM, the percentage of apoptotic cells is related to the dosage used to generate the RCM (Fig. 3). These results suggest that, at least for doses ranging from 0.5 to 4 Gy, the cytotoxic soluble factor released into the culture medium capable of triggering apoptosis must be directly dose related.

Fractionation schedule and bystander response

Most RT modes include dose fractionation [20,42]. Bystander effects may complicate this because the multiple-field approach could increase the systemic burden of bystander factors. Our results (Fig. 2D) suggest that the cumulative effect of exposure to 4 cycles of treatment with a medium from cells exposed to 6 Gy could be described by a non-interactive situation in which each fraction exerts its own individual effect. Whether this result is due to the recipient cells responding to each fresh signal without any adaptation or any ‘recovery’ between signals remains unknown. What is clear is that with this strategy we could overcome the bystander saturation limit observed for cells receiving one single treatment with RCM produced with cells irradiated over a wide range of doses (Fig. 2A). This saturation means that for any given end-point not every cell responds with a bystander effect. As far as damage induced by RCM is concerned, previous work has indicated that cells in the S phase may be more vulnerable to bystander effects [43]. These results, together with: (i) the well known effect of cell hypersensitivity at dosage levels of less than 1 Gy, typically around 0.5 Gy [44–47]; (ii) our finding that the values of \( K_{by} \) for bystander-induced cell death are around 0.2–0.3 Gy; (iii) the recently published data that one of the molecules that plays a part in bystander cell death is TRAIL [48,49]; and (iv) the fact that TRAIL has a short half life in plasma [38], prompted us to suggest a change in the dose-fractionation schedule [20] in an attempt to unite hypersensitivity, maximum extracellular concentration of death receptor ligands, the upregulation of death receptors in tumor cells and cell redistribution in the tumor after radiotherapy.

What might the nature of the soluble factor present in RCM be?

The bystander effect is a manifestation of radiation-induced signals that travel from irradiated cells to their neighbors and by molecular mediators that are constitutively expressed or induced in target cells [22.50–54], which might be either specific or non-specific in their mode of action, and within this context diffusible oxygen reactive species and secreted factors have been identified as possible mediators in the indirect bystander effect [12]. We have also shown that dilution experiments with RCM indicate a dose-response effect (Fig. 2C). Apart from this, several recent studies have revealed that one or more factors secreted by irradiated cells, such as TGF-β, interleukin-8, TNF-α and TRAIL, may be involved in the propagation of the effect [28,47,48,52–58]. Our results show consistent cell death caused by RCM in both tumor cell models (Fig. 2A), which suggests that these cytokines may be secreted by irradiated cells and that these may be the effectors of the cell death observed here. Furthermore, our findings (Fig. 3B) confirm recently published results revealing that UCSSCs are insensitive to RCM in the same way that mesenchymal stem cells from bone marrow, adipose tissue and umbilical cord are [48]. Thus, we can confirm
that radiation-induced signaling in non-targeted bystander cells differs in several key aspects from the response of directly irradiated cells whatever the radiation-induced lesion that gives rise to the bystander effects may be. In addition, it now seems clear that because of bystander effects radiation effects go far beyond the expected reduction in the number of viable cancer cells. In contrast to melanoma cells, mesenchymal stem cells are not targeted by the Fas, TRAIL and TNF-α ligand signal molecules produced by radiation-activated tumor cells.

Our results support the hypothesis that cell therapy with UCSSC cells in combination with radiotherapy could be of great interest because these cells are able to host in primary tumors and microscopic tumor deposits [59,60] and liberate TRAIL in response to radiation treatment.

The linear–quadratic (LQ) model and its mechanistic interpretation adequately describe cell response to radiation above 2 Gy. At doses below 2 Gy the LQ model substantially underestimates the effect of irradiation. Although this was first observed in 1993 [31] the molecular mechanism involved in the low-dose hyper-radiosensitivity model [61] and in the bystander effect has not yet been fully determined.

DNA damage model and non-targeted model after radiotherapy

We describe here an empirical model of cell radiation response that incorporates both local and non-local effects. A mathematical framework for separating the direct and bystander components of cell-radiation dose–response has recently been put forward [62]. Evidence now shows that, as well as these direct DNA damage-dependent effects, irradiated cells also send signals to their neighbors; here we consider that clonogenic cell survival (S) after radiation treatment depends on both the effects of pure radiation interaction (ZR) and bystander interaction (ZBy). Assuming that the radiation and bystander effects on cell survival are independent, the total response might be expressed as:

$$S = Z_R \cdot Z_{By}$$

Our experimental results have allowed us to calculate the S values according to the surviving fraction after each dose (dose range 0–8 Gy) from clonogenic cell survival experiments ZR (Fig. 4, red lines) and the values of ZBy from the results of RCM transfer experiments (Fig. 4, black lines) for the same dose range. The results of these calculations are plotted (S) in Fig. 4 (blue lines). The results corresponding to G361 and A375 appear in the upper panel. The curves plotted allow us to confirm that the shape of the theoretical values (S) produced by our approach for separating the direct and bystander components of cell response to radiation closely matches that of hypersensitivity at low doses. Thus, from 2 to 8 Gy the differences between the S and ZR curves are apparently constant and the lines are parallel, whereas in the range from 0 to 2 Gy these differences increase slightly at first to reach a maximum value at around 1 Gy and then decrease until they adopt a constant slope in the second part of the S curve (Fig. 4, lower panel).

Anyway, we can arrive at a theoretical estimation of the overall effect of radiation by measuring the ZR and ZBy values and calculating the composed survival probability, S. The mathematical expression of our final model is

$$S = e^{-(\alpha D + \beta D^2)} \cdot \left(1 - \frac{D}{K_{By} + D}\right)\left[\nu\right]$$

This means that the probability of clonogenic cell survival depends on both the direct radiation effect (the linear-quadratic model) and bystander effects, these effects also being a phenomenon composed of long- and short-range bystander actions, the relative importance of which, at least in vitro, could be estimated. The final values of S indicate that the lethal effects of radiation on tumor cells can be significantly increased by unexpected interactions between irradiated and non-irradiated bystander cells.
We have found that the death of bystander cells would fit a model in which cell death seems to be the result of the expression of functional death receptors in the non-irradiated tumor cells and the secretion of soluble forms of death receptor ligands. The role of cell-death receptors and their ligands in apoptotic cell death is, on the other hand, very well known. In our study, we have found that both phenomena might be exploited clinically if it is practicable to deliver radiotherapy at doses of less than 1.6 Gy per fraction, twice a day, 8–12 h apart, to escalate the total dose compared to conventional fractionation. The aim would be to take advantage of the extra radiosensitivity in the HRS region, the additional bystander cell death and the adaptation of dose fractionation treatment to the mean life of cytokines in the vascular space.

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References

[25] Azzam EI, de Toledo SM, McMillan TJ, Little JB. The bystander component of cell death measured in this work highlights the importance of confirming our observations in a clinical situation. Furthermore, the UCSSCs used here proved to be resistant to bystander effects and may thus be suitable tools for TRAIL delivery to tumors, as has been demonstrated recently by other researchers.
[26] The hyper-radiosensitivity at low doses together with the bystander effect observed at the same dose lead us to suggest that both phenomena might be exploited clinically if it is practicable to deliver radiotherapy at doses of less than 1.6 Gy per fraction, twice a day, 8–12 h apart, to escalate the total dose compared to conventional fractionation. The aim would be to take advantage of the extra radiosensitivity in the HRS region, the additional bystander cell death and the adaptation of dose fractionation treatment to the mean life of cytokines in the vascular space.
Bystanders effects in radiation therapy


