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The non-targeted effects of radiation are perpetuated by exosomes

Ammar Al-Mayah[a], Scott Bright[a], Kim Chapman[a], Sarah Irons[b], Ping Luo[c], David Carter[d], Edwin Goodwin[e], Munira Kadhim[a,*]

[a] Genomic Instability Group, Oxford Brookes University, Gipsy Lane Campus, Headington, Oxford OX3 0BP, United Kingdom
[b] Insect Virus Research Group, Oxford Brookes University, Gipsy Lane Campus, Headington, Oxford OX3 0BP, United Kingdom
[c] Izon Science Ltd., The Oxford Science Park, Magdalen Centre, Robert Robinson Avenue, Oxford OX4 4GA, United Kingdom
[d] Chromatin and non-coding RNA, Oxford Brookes University, Gipsy Lane Campus, Headington, Oxford OX3 0BP, United Kingdom
[e] The New Mexico Consortium, Los Alamos, NM 87544, USA

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ABSTRACT

Exosomes contain cargo material from endosomes, cytosol, plasma membrane and microRNA molecules, they are released by a number of non-cancer and cancer cells into both the extracellular microenvironment and body fluids such as blood plasma.

Recently we demonstrated radiation-induced non-targeted effects [NTE: genomic instability (GI) and bystander effects (BE)] are partially mediated by exosomes, particularly the RNA content. However the mechanistic role of exosomes in NTE is yet to be fully understood.

The present study used MCF7 cells to characterise the longevity of exosome-induced activity in the progeny of irradiated and unirradiated bystander cells. Exosomes extracted from conditioned media of irradiated and bystander progeny were added to unirradiated cells. Analysis was carried out at 1 and 20/24 population doublings following medium/exosome transfer for DNA/chromosomal damage. Results confirmed exosomes play a significant role in mediating NTE of ionising radiation (IR). This effect was remarkably persistent, observed >20 doublings post-irradiation in the progeny of bystander cells. Additionally, cell progeny undergoing a BE were themselves capable of inducing BE in other cells via exosomes they released. Furthermore we investigated the role of exosome cargo. Culture media from cells exposed to 2 Gy X-rays was subjected to ultracentrifugation and four inoculants prepared, (a) supernatants with exosomes removed, and pellets with (b) exosome proteins denatured, (c) RNA degraded, and (d) a combination of protein–RNA inactivation. These were added to separate populations of unirradiated cells. The BE was partially inhibited when either exosome protein or exosome RNA were inactivated separately, whilst combined RNA–protein inhibition significantly reduced or eliminated the BE. These results demonstrate that exosomes are associated with long-lived signalling of the NTE of IR. Both RNA and protein molecules of exosomes work in a synergistic manner to initiate NTE, spread these effects to naïve cells, and perpetuate GI in the affected cells.

1. Introduction

Carcinogenesis proceeds through the accumulation of genetic and epigenetic changes that include deactivation of tumour suppressor genes and activation of oncogenes [1]. The microenvironment in which a genetically altered cell resides also plays several important roles. It can suppress the expression of malignant cell characteristics, promote cell division, or increase the mutation rate [2]. Radiation has proven to be valuable in the study of cell–cell communication, such as that which occurs within the microenvironment, and may include signals passed between normal cells, from normal to malignant cells or vice versa, or between malignant cells. One important class of responses to cell–cell communication signals is referred to as "non-targeted" because the effects arise in cells that were not themselves exposed to radiation. Non-targeted effects (NTE) of ionising radiation occur both in the progeny of irradiated cells, in which it is called genomic instability (GI), and in cells that are in contact or close proximity to irradiated cells but have not been themselves exposed, the so-called bystander effect (BE) [3–5].

Bystander cells and genetically unstable cells exhibit biological responses similar to directly irradiated cells including genetic

* Corresponding author. Tel.: +44 01865 483954; fax: +44 01865 483242.
E-mail address: mkadhim@brookes.ac.uk (M. Kadhim).

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mutations, chromosome damage, micronuclei and apoptosis. The effect is mediated through gap junction intercellular communication (GJIC) where small protein channels link adjacent cells and allow the passage of signalling molecules. Alternatively BE can be mediated through endocytosis whereby an irradiated cell has released a signalling molecule which is endocytosed by a cell spatially removed from the signals' point of origin. The nature of the soluble transmitting factor(s) is yet to be fully understood, but cytokines including IL-8 [6,7]; TGF-beta [8,9] and TNF-alpha [10,11], as well as calcium fluxes, NO [12] and ROS [13] have been suggested as mediators of bystander responses. A role for plasma membrane-bound lipid rafts has also been indicated [14]. Several mechanistic studies have shown an important role for the NF-κB-dependent gene expression of *interleukin 8*, *interleukin 6*, *cyclooxygenase-2*, *tumour necrosis factor* and *interleukin 33* in bystander cells. These studies suggest directly irradiated cells produce the cytokines and prostaglandin E2 by means of autocrine/paracrine mechanisms, which in turn further activate signalling pathways [15]. From gene expression and gene ontology analysis, NF-κB forms a major transcriptional hub in bystander cells in contrast to directly irradiated cells where p53 drives transcriptional responses to a direct DNA damage signal [16]. Furthermore, other work by Albanese and Dainiak [17] showed that irradiated cells shed vesicles such as exosomes or radiation intercellular signal exosomes which affect the recipient cells [18–21]. Recently, microRNA (miRNA) has been shown to be a potential mediator of BE [22–24].

Exosomes are small (<150 nm) [53] membrane-bound vesicles that are released by cells into the extracellular environment [25]. Exosomes are formed by inward budding of the endosomal membrane which subsequently engulfs cytoplasmic material including protein and RNA [52]. Exosomes are held within the endosome until targeted for destruction by the lysosome or released into the extracellular environment. After release they are endocytosed by a recipient cell, however full translocation across the membrane is not always necessary to induce an effect [26].

Exosomes have a broad spectrum of functions in health and disease including, expulsion of unwanted material, propagation of pathogens, modulation of the immune system, antigen presentation, and perhaps most relevant to this paper, intercellular communication [27]. The variety in function of exosomes is a result of the cell type and conditions of release. For example, cancer cells secrete exosomes that appear capable of inducing oncogenic properties in recipient cells, including increased cell division or metastatic behaviour [28–30]. Consistent with findings that exosome levels are raised in the blood of cancer patients [29,31], exosome-mediated signalling may underlie the cancer 'field effect', in which tumour cells have been shown to influence the phenotype of nearby cells [32].

Only recently light has been shed on the subcellular compartments and mechanisms involved in their biogenesis and secretion opening new avenues to understand their functions [27].

Recent work [33] has demonstrated that exosomes released from irradiated cells, may be responsible at least in part for BE in MCF-7 breast cancer cells arising through exosomal intercellular communication. Furthermore, treatment of bystander cells with exosomes isolated from this media increased the levels of genomic damage. These results suggest that the bystander effect, and genomic instability, are at least in part mediated by exosomes and implicate a role for RNA.

The current study was established to investigate the longevity of exosome BE-inducing activity in the progeny of irradiated and bystander cells, and also to determine the role of exosomes' RNA and protein molecules in this process. In addition, to further characterise exosomes released from irradiated cells, the size and concentration of the exosomes were measured using tuneable resistive pulse sensing (TRPS).

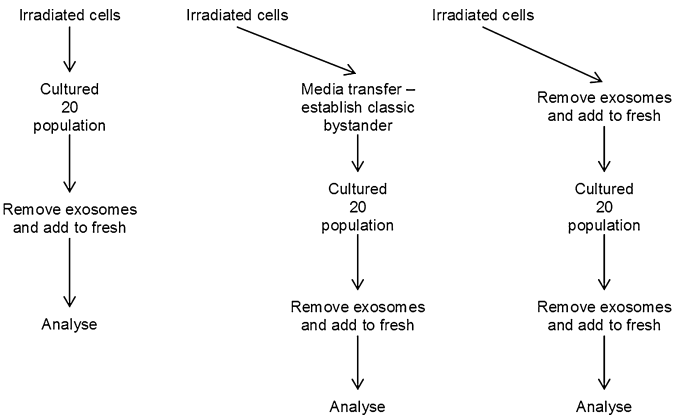

Fig. 1. Schematic of exosomes extraction from the progeny of irradiated, bystander, and exosome bystander cells for groups created below.

2. Materials and methods

2.1. Cell culture and experimental design

MCF7 breast epithelial cancer cells were utilised in this study and were cultured as described previously in Al-Mayah et al. [33].

2.2. The longevity of exosome-mediated BE-inducing activity in the progeny of irradiated and bystander cells

Experiments were set up as described in Fig. 1. MCF7 cells were grown in T75 flasks. Cells were exposed at 70% confluence to 2 Gy X-ray irradiation. Flasks were incubated at 37 °C following irradiation for 4 h, after which, the irradiated cell conditioned media (ICCM) were pooled and filtered through 1% BSA treated 0.2 μm filter. Following ultracentrifugation [33], two different bystander groups were established. For the classic bystander group, supernatant from irradiated cell (ICCM) with cell debris removed was added to fresh MCF7 cells at 70% confluence. For the exosome bystander group, the ICCM was ultra-centrifuged, the exosomes' pellets collected and transferred to un-irradiated cells for establishing the exosome bystander group (ICCM exosomes group). Control cell conditioned media (CCCM) was also established in parallel for each corresponding group by repeating the procedure above without irradiation. In addition, a group of directly irradiated cells were established within the same experiments. Cells from each group were cultured and propagated until p10 (approximately 20 cell-doublings). In order to determine activity of the exosomes in the progeny of irradiated and all the bystander groups, after 20 population doublings following irradiation/bystander procedure, exosomes from progeny media of irradiated, classic bystander, bystander exosome, and their corresponding control groups were purified and transferred to fresh MCF7 cells in order to investigate whether the exosomes of these groups are able to induce DNA damage in fresh un-irradiated cells using the comet assay as described in Al-Mayah et al. [33]. Cells were analysed using Komet 5.5 Image Analysis Software (Kinetic Imaging Technology/Andor, Germany). For bystander conditions the total exosome fraction was added to recipient cells.

2.3. Determine the role of exosomes' RNA and protein molecules in the process of NTE induction

To investigate the possible role(s) of exosomes' cargo (RNA and protein molecules) in the induction of NTE, experiments were set up