The Emerging Role of Exosomes in the Biological Processes Initiated by Ionizing Radiation

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Submitted July 8, 2017; revised version submitted January 18, 2018

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Running title: Exosome signaling in the biological processes initiated by radiation.

ABSTRACT

The biological effects of radiation can be divided into targeted and non-targeted depending on whether or not radiation energy has been absorbed by a cell under observation. Non-targeted effects (NTEs) are the result of intercellular communication between irradiated cells and unirradiated (*i.e.* non-targeted or bystander) cells. The impact of NTEs on radiation risk is not well understood for simple radiation exposure scenarios and even less so for complex radiation fields such as those outside of earth's atmosphere. Here we review the emerging role of extra-cellular vesicles, in particular exosomes, in the biological processes responsible for NTEs. Exosomes carry various cargo such as nucleic acids, proteins and metabolites, and they are exchanged between cells giving them the capability to act as intercellular messengers in the initiation of NTEs. Ionizing radiation modulates release of exosomes and the cargo they contain, and these exosomes induce functional changes in recipient cells. There are, however, large gaps in our current knowledge, particularly relating to altered exosomal loading following radiation as well as the functional effects induced by exosomes in recipient cells. A better understanding of NTEs, including the role of exosomes, will be required before they can be incorporated into radiation risk assessment or mitigation of radiation effects.

INTRODUCTION

The classical understanding of radiation biology centers around damage to a biological target through direct traversal by radiation, usually considered to be nuclear DNA. Ionizing radiation can damage DNA resulting in a DNA damage response that can either faithfully repair the damage or misrepair the damage

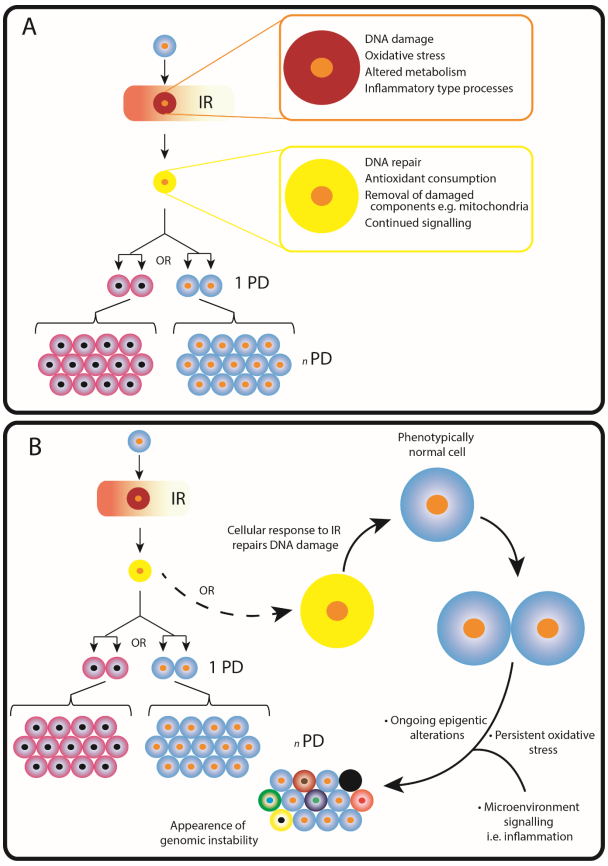

Figure 1 (A and B)

Figure 1: Basic overview of responses to radiation. A. Classical theories identified radiation can cause extensive damage to nuclear DNA. If the damage is too complex the cell may commit to cell death; alternatively there are a number of repair mechanisms by which to correct this damage. If the cell correctly repairs this damage it is expected to divide and proliferate as normal. However, in some cases the damaged DNA is repaired incorrectly introducing mutations or gross chromosomal aberrations. As these cells divide, the mutation or chromosomal aberration is passed on to both daughter cells and is present across all the progeny and is said to be clonal. B. Non-targeted effects add to this theory where clonal damage can still occur but other processes are ongoing that lead to GI. Initially for some undefined period of time the cells appear normal. However, with time, the cell population may develop a range of aberrations that are not consistent across the progeny indicating that they are likely to have occurred de novo to that cell cycle following the first cell division. The increased rate at which de novo aberrations arise is defined as genomic instability.

Radiation-induced genomic instability (GI) is a genome-wide process. It is characterized by an increased rate of cytogenetic abnormalities, mutations, gene amplifications, transformation, and cell death in the progeny of irradiated cells many generations after the initial insult. The characteristics of the instability depend on several factors including cell and tissue genetic background, radiation type, dose and dose rate, and the test system being studied (*5-8*). The mechanisms behind this process involve epigenetic factors (*9-12*), oxidative stress and inflammatory signals (*13-20*). There is also evidence to suggest bystander effects have the potential to induce GI in some circumstances (*5, 18, 21-24*). Bystander effects have been documented in several experimental

systems both in vitro and in vivo (*25, 26*) and have been observed following a variety of radiation types, doses and exposure protocols, including those more like space radiation than the alpha particles used in early work (*27, 28*). Bystander Effects are also triggered by very low doses, as low as a single high-LET particle (*29-32*), and have an off-to-on dose response without further increase in measured effects above the triggering dose. Bystander Effects can recruit naïve cells into expressing the same response phenotypes as that of irradiated cells, despite the cells never having being traversed by IR; instead the effects are induced through the transfer of a signal from irradiated to unirradiated cells (Figure 2) (*33*). The causative signals are likely to include cytokines, reactive oxygen species and epigenetic regulators such as miRNA (*34-37*). Irradiated cells can distribute one or more signals through two routes; firstly, between adjacent irradiated and unirradiated cells linked by gap junctions (*38*), and secondly through secretion of signaling molecules into the extracellular environment (*39*). The latter has recently been shown to involve the transfer of signals through extracellular vesicles (EVs) (*40*).

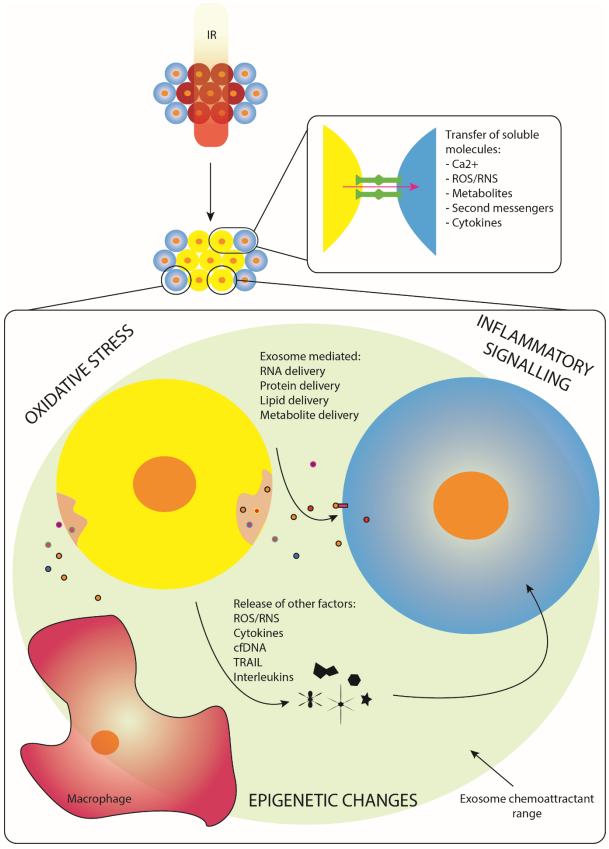

Figure 2

Figure 2 shows the transfer of signals from irradiated cells to unirradiated cells (bystander effects). The box showing transfer of soluble molecules shows transfer between cells connected by gap junctions but this is not always the case. Many studies using irradiated cell conditioned media (ICCM) (i.e. no gap junctional communication) have shown the transfer of soluble molecules between irradiated and unirradiated cells.

In this review, we focus on EVs, in particular exosomes, as delivery vehicles for bystander signals and the downstream effects they have on neighboring cells with relevance to radiation-induced NTEs.

EXTRACELLULAR VESICLES

EVs are abundant in both *in vitro* cell culture systems as well as *in vivo* fluids including, amongst others, plasma, urine and saliva. Several subcategories of EVs exist including microvesicles, apoptotic bodies and exosomes. As shown in Table 1, the characteristics and attributes that define these vesicles are primarily based on size and presence or absence of certain biochemical markers (*41-44*).

Exosomes are a class of EVs secreted by several cell types into the extracellular environment. The discovery of various important cargo such as mRNA, miRNA, DNA, protein , metabolites, lipids and other non-coding RNAs sparked interest in their function (*45, 46*).

Exosomes are produced in the endosomal network; their membranes are composed of lipids and proteins (*47*). The proteins identified in exosomes are representative of endosomes and the plasma membrane but show very little overlap with other organelles such as the nucleus or Golgi apparatus. Despite their endosomal origin it is difficult to distinguish biochemically between plasma membrane-derived vesicles and exosomes (*48*). The lipid membrane of EVs shows a distinct profile compared to conventional plasma membranes including enrichment in sphingomyelin, cholesterol, ceramide and phosphatidylserine, with some documented reductions in phosphatidylcholine (*48-50*).

Exosomes cargo is selected for export rather than being a simple consequence of what is present in the cell at that time. Upon the appropriate cues these vesicles are shuttled to the cell membrane and released into the extracellular environment. (*51-54*). Exosomes can be taken up by neighboring cells in the immediate vicinity or transported via the blood stream to distant sites. Cells take up exosomes through a variety of processes and cargo is released within the recipient cell, with several studies showing this cargo is often functional within the recipient cell (*55-60*).

A consensus is beginning to develop around exosomes as mediators of intercellular communication in carcinogenesis and metastasis (*61*), with the apparent ability to promote angiogenesis, alter fibroblasts to become cancer-associated fibroblasts, sequester anticancer therapeutics, modulate the immune system, and alter distant microenvironments to help circulating cancer cells establish a metastatic site (*62, 63*). It is still not completely clear how their function changes with cell type and the stresses put on the cell (*19, 64, 65*).

Table 1: Extracellular vesicle descriptions in the literatures

Name	Description	Reference
Apoptotic bodies	They are variable size, 500– 2000 nm in diameter; consist of cytoplasm with tightly packed organelles. Extensive plasma membrane blebbing occurs followed by karyorrhexis and separation of cell fragments during a process called 'budding'.	(*66, 67*)
Ectosomes	Membrane microvesicles derived from neutrophils or monocytes. They vary from 100 to 1000 nm. The doubt is whether vesicles larger than 350-400 nm are true ectosomes. They are small shedding membrane vesicles, budded directly from cell membrane.	(*68, 69*)
Exosomes	They are homogenous membrane bound vesicles with size of 40-100nm in diameter. They are derived from the endocytic recycling pathway. In endocytosis, endocytic vesicles form at the plasma membrane and fuse to form early endosomes. These mature and become late endosomes where intraluminal vesicles bud off into an intra-cytoplasmic lumen. Instead of fusing with the lysosome, these multivesicular bodies directly fuse with the plasma membrane and release exosomes into the extracellular space. CD63, CD9 and TSG101 can be considered as exosomal markers.	(*70-73*)

EXOSOMES AND RADIATION-INDUCED BYSTANDER EFFECTS

The fact that exosomes can have such potent effects in a diverse and complicated environment poses interesting questions in relation to radiation biology, some of which are especially pertinent to the complex mixture of radiation types of interest to space exploration. For example:

(1) Is exosome number dependent on cell type, radiation quality, dose rate, or total dose?

(2) How does exosomal loading change under the conditions above.

(3) Does exosome-mediated communication offer a mechanistic link between the bystander effect and GI?

(4) To what extent does the radiation release of exosomes contribute to the cellular response to radiation exposure and ultimately to the risk of developing cancer?

Exosome-related research within radiation biology is a relatively new field; however research already shows exosomes are an essential component of the cellular response as detailed below. Ionizing radiation is a known activator of p53 (*25*); p53 can also function to regulate exosome release (*26, 74, 75*). This suggests p53 might regulate exosomes under cellular stress such as caused by ionizing radiation. Exosome uptake is also increased in response to radiation through the formation of CD29/CD81 complexes (*65*). However, it is likely that alteration in the cargo may also be essential in inducing bystander effects. Al-Mayah et al. 2012 were some of the first to explore this when they documented that exosomes from irradiated MCF7 cells carried bystander signaling molecules capable of inducing early and persistent chromosomal damage, and provided evidence the signal is mediated through exosomal protein and RNA. Since then work has been ongoing to further explore exosome contents as potential inducers of bystander effects (*76*). There is evidence to suggest a variety of contents are responsible for exosome-mediated radiation-induced bystander effects. The miRNAs have been most extensively studied; they are small RNA sequences approximately 22 nucleotides in length. They have been implicated in a number of processes, particularly gene expression, providing an epigenetic form of post-transcription gene regulation (*77*). They are able to affect a number of responses such as metabolism, immune response, proliferation, differentiation and migration. miRNAs are highly abundant in exosomes, with one report suggesting 42% of nucleic acid content was miRNA with the remainder composed of other types of RNA including ribosomal, piwi interacting and transfer (*78*). Exosome miRNA content also differs from that of the parent cell, suggesting the loading process is not random and certain miRNAs are actively sorted into exosomes (*79*). How loading changes in response to external stimuli such as ionizing radiation is under investigation and will be important to consider for radiation-induced NTE.

Research into the exosome-mediated bystander effect has started to investigate exosomal miRNA's and the associated pathways with which they interact (*40, 76, 80-83*). Exosomes isolated from cells irradiated with ^{60}Co γ-rays at a dose rate of 1.98 Gy/min at room temperature showed several enriched miRNA's including miR-7-5p (*84*). This miRNA was shown to influence the EGFR/Akt/mTOR pathway in the recipient human bronchial epithelial cell line BEP2D, ultimately increasing levels of autophagy. Transfer of miR-21 in exosomes from irradiated cells has also been documented. Increased micronuclei formation was attributed in part to the effect of miR-21 on its gene targets. Although no mechanism for micronuclei induction was presented, there is evidence elsewhere that miR-21 is a potent onco-miR (*85, 86*) and its expression can remove control at the G1/S phase cell-cycle transition; this could potentially account for the formation of micronuclei (*40, 76, 80-82*)..

Exosomal miRNA transfer and associated bystander effects have also been observed *in vivo* by Tang *et al*. (2016), who reported differential miRNA expression profiles from patients pre/post radiotherapy. Nine miRNAs were downregulated while one, miR-208a, was significantly upregulated. Exosomes from irradiated cells enriched for miR-208a were shown to be internalized by the A549 cell line. The delivery of miR-208a induced a radioresistant phenotype likely through its effects on the p21/Waf1 pathway (*87*). Others have also observed a prosurvival effect induced by exosomes from irradiated cells, this time thought to be elicited through triggering DNA repair (*57*).

miRNA is not the only component of exosomes capable of inducing bystander effects. Arscott *et al*. (2013) identified exosomes from irradiated cells as having the ability to induce functional changes in unirradiated bystander cells through mRNA and protein delivery. The imported mRNA transcripts coded for proteins associated with cellular movement such as CTGF. As a result protein levels of CTGF also increased as did cell migration (*88*).

Other nucleic acids such as long noncoding RNA (lncRNA) have been found to be associated with exosomes. The lncRNA are RNA molecules in excess of 200 bp and do not code for a protein (*89*); however, they have been shown to be involved in processes such as protein synthesis, RNA maturation and even regulating chromatin structure (*89-91*). lncRNA named "PARTICLE" increased after low dose

exposure at the 24 hour post-irradiation timepoint. PARTICLE repressed the expression of MAT2A by controlling the methylation status of its upstream promotor CpG islands (*92*). This highlights the ability of exosomes from irradiated cells to act epigenetically in bystander cells, a mechanism that could be crucial in understanding bystander effects and their links to genomic instability, reviewed by Hewson and Morris in 2016 (*93*).

Exosomes are also known to carry protein. Exocarta has listed 41,860 entries for proteins in exosome fractions. In terms of functional effects, Jelonek *et al*., (2015) performed a proteomic analysis on exosomes from control and irradiated FaDu cell line derived from human head and neck squamous cell carcinoma (HNSCC) with a 2 Gy dose of 6 MeV photons using the linear accelerator Clinac 600 and incubated for 18 hours before exosome isolation. They found 236 up-regulated proteins and 69 down-regulated proteins. These proteins were annotated with gene ontology (GO) terms, the up-regulated terms included "mRNA metabolism", "viral process", "RNA metabolic process", and "DNA damage response, signal transduction by p53 class mediator resulting in cell cycle arrest" (*94*) . Specific functional changes have also been observed by Baluch *et al*., (2016) who demonstrated that microvesicles from irradiated cells could increase matrix metalloproteinase activity in unirradiated cells which increased their invasiveness, although not through direct transfer of the enzymes themselves (*95*). Other investigations in *ex vivo* peripheral blood mononuclear cells (PBMCs) have shown a difference in exosome protein content post irradiation with 60 Gy γ-rays; exosome release was increased by three-fold, the differential expression however was relatively modest with approximately 10 proteins showing increased presence following irradiation (*26*), albeit this was performed with 2D electrophoresis which offers limited resolution compared to more sophisticated mass spectrometry. Recent studies reported that exosomes contain genomic DNA . This exosomal DNA (exoDNA) represents the entire genome, and for tumors it reflects the mutational status of parental cancer cells (*83, 96*). These findings suggest that (exoDNA) can be used to identify mutations present in the parental tumor, thus illustrating significant translational potential as a circulating biomarker for cancer in the clinic. However, the mechanism of how DNA enters

the exosomes is as yet unknown, and the feasibility of using exosomal DNA in diagnosis and therapy of cancer has yet to be demonstrated.

CONCLUSIONS

The mechanisms of ionizing radiation-induced NTEs are not as yet fully understood. It is, however, known that 1) exosomes play an essential role in delivering signals from irradiated cells to naïve bystander cells, and 2) exosomes produced and released from initiated cells further spread the effects of radiation by initiating other naïve bystander cells. Additionally the evolution from a short-term bystander effect into persistent GI is accompanied by exosome production in the progeny of cells expressing GI (*40, 76*). This latter observation suggests that exosome production, release, and internalization may be at least partially responsible for perpetuating an unstable phenotype. The cargo carried within exosomes can act through a variety of mechanisms including proinflammatory signaling, modification of the extracellular environment, and epigenetics within the cell. These processes are important to understand in the context of radiation biology. Exosome-mediated intercellular communication has only been investigated to a limited extent in radiation-induced NTEs, with both exosomal protein and RNA being implicated in bystander effects at both early and delayed time-points.

Whether exosomes have roles in the bystander effect and GI that extend beyond signaling is less clear. Exosomes and extracellular vesicles are known to carry numerous other contents such as metabolites, amino acids and lipids. These molecules have been shown to induce functional effects in recipient cells, for example metabolic switching after the delivery of metabolites, or induction of inflammation after lipid delivery (*58*). These aspects have yet to be explored in relation to radiation biology.

Although seminal research on genomic instability and bystander effects was conducted using high LET alpha particles, studies of exosomes in the radiation response are currently limited to low LET photons. In contrast, the space radiation environment is composed mainly of high and low LET energetic

charged particles. Given this gap in our knowledge, a focused research effort is required to answer such questions as:

- Once initiated, are NTE the same regardless of the initiating radiation type?
- Is the threshold for an initiating dose dependent on LET or the radial dispersion of energy deposition along charged particle tracks through cells and tissues (*i.e.* track structure)?
- How do LET, track structure, dose and dose rate affect exosome packaging and release?

Also required are quantitative models capable of linking initiating events – both physical (radiation) and molecular (intercellular signaling) – to biological responses. These models will be essential for incorporating NTE into radiation risk assessment and for evaluating the efficacy of countermeasures. Further, testing these models will challenge experimentalists to acquire datasets that are far more quantitative than currently available. In conclusion, the investigation of NTE has made substantial progress since their discovery but we are not yet positioned to evaluate potential health effects of NTE resulting from space radiation exposure. Achieving this goal will require a better understanding of the basic biology of NTE including exosome signaling, the dependence of NTE on radiation characteristics, and quantitative models of NTE.

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