Biochimica et Biophysica Acta xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Review

Calcium, oxidative stress and connexin channels, a harmonious orchestra directing the response to radiotherapy treatment?

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ARTICLE INFO

Article history:
Received 30 November 2016
Received in revised form 2 February 2017
Accepted 4 February 2017
Available online xxxx

Keywords:
Ionizing radiation
Calcium
Oxidative stress
Connexin hemichannel
Gap junction
Bystander effect

ABSTRACT

Although radiotherapy is commonly used to treat cancer, its beneficial outcome is frequently hampered by the radiation resistance of tumor cells and adverse reactions in normal tissues. Mechanisms of cell-to-cell communication and how intercellular signals are translated into cellular responses, have become topics of intense investigation, particularly within the field of radiobiology. A substantial amount of evidence is available demonstrating that both gap junctional and paracrine communication pathways can propagate radiation-induced biological effects at the intercellular level, commonly referred to as radiation-induced bystander effects (RIBE). Multiple molecular signaling mechanisms involving oxidative stress, kinases, inflammatory molecules, and Ca^{2+} are postulated to contribute to RIBE. Ca^{2+} is a highly versatile and ubiquitous second messenger that regulates diverse cellular processes *via* the interaction with various signaling cascades. It furthermore provides a fast system for the dissemination of information at the intercellular level. Channels formed by transmembrane connexin (Cx) proteins, i.e. hemichannels and gap junction channels, can mediate the cell-to-cell propagation of increases in intracellular Ca^{2+} by ministering paracrine and direct cell-cell communication, respectively. We here review current knowledge on radiation-induced signaling mechanisms in irradiated and bystander cells, particularly focusing on the contribution of oxidative stress, Ca^{2+} and Cx channels. By illustrating the tight interplay between these different partners, we provide a conceptual framework for intercellular Ca^{2+} signaling as a key player in modulating the RIBE and the overall response to radiation.

1. Introduction

Cancer is recognized as one of the main causes of morbidity and mortality. It accounts worldwide for more than 14 million new cases and more than 8 million deaths each year. The number of diagnosed cases is expected to more than double during the next two decennia [1]. A variety of cancer treatment options exist with treatment recommendations varying upon the tumor type and stage. The mainstays of cancer therapy are surgery, chemotherapy and radiotherapy. At least 50% of all diagnosed cancer patients receive radiotherapy at some point during the cancer treatment, resulting in a cure rate of about 40% [2]. Moreover, the number of patients in Europe who require radiotherapy treatment is expected to increase with 16% from 2012 to 2025 [3]. The current treatment strategy is, however, far from optimal with the success rate of radiotherapy being impaired by both the radiation resistance of tumor cells and off-target effects on healthy tissue [4,5]. The latter are not only the number 1 cause of healthy tissue injury, but

Abbreviations: ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; $[Ca^{2+}]_i$, intracellular calcium concentration; CaM, calmodulin; CaMKII, Ca^{2+}/calmodulin-dependent kinase II; cAMP, cyclic adenosine monophosphate; COX-2, cyclooxygenase-2; Cx, connexin; EGFR, epidermal growth factor receptor; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; GJC, gap junction channel; GPCR, G-protein coupled receptor; GRP75, glucose regulated protein 75; HMGB-1, high-mobility group box 1; IL, interleukin; iNOS, inducible nitric oxide synthase; IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptor; MAPK, mitogen-activated protein kinase; mtDNA, mitochondrial DNA; mTOR, mammalian target of rapamycin; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; nNOS, neuronal nitric oxide synthase; Panx, pannexin; PGE2, prostaglandin E2; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PLC, phospholipase C; P_2X_7R, P_2X_7 receptor; RIBE, radiation-induced bystander effect; RNS, reactive nitrogen species; ROS, reactive oxygen species; RyR, ryanodine receptor; TNF-α, tumor necrosis factor-α; VDAC, voltage-dependent anion channel.

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http://dx.doi.org/10.1016/j.bbamcr.2017.02.007
0167-4889/

also of cancer initiation and promotion. Major efforts are currently focusing on the optimization of new strategies to combat cancer such as gene therapy, immunotherapy or molecular-targeted therapy, or to improve the therapeutic gain of the conventional strategies [6,7].

In recent years, connexin (Cx)-based intercellular communication has emerged as an attractive target to modulate the response to cancer therapy [8,9]. Connexins (Cxs) constitute a family of transmembrane proteins that, by forming channels, offer two intercellular signaling pathways; (i) a direct transfer of ions and small molecules (<1.5 kDa) between the cytoplasm of neighboring cells *via* gap junction channels (GJCs) and (ii) a pathway for paracrine communication *via* the exchange of molecules between the cytoplasm of an individual cell and its extracellular environment through hemichannels [10,11]. Cx channels have been shown to affect the response to radiotherapy, chemotherapy and gene therapy [8,9].

An opportunity of exploiting Cx channels in cancer therapy lies within the so-called bystander effect [8,12]. In essence, the bystander effect refers to the transmission of responses from cells exposed to a certain stimulus, to non-targeted neighboring or more distant cells by means of intercellular communication. We previously reported that both hemichannels and GJCs composed of Cx43 provide potent pathways for the propagation of an apoptotic stimulus, thereby expanding cell death to adjacent and remote healthy cells within a brain glioma cell culture. The propagation mechanism relies on the intercellular transfer of the Ca^{2+}-mobilizing messenger inositol 1,4,5-trisphosphate (IP_3) that leads to the cell-to-cell propagation of increases in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), commonly referred to as intercellular Ca^{2+} waves [13,14]. At present, there is considerable evidence that similar mechanisms of intercellular communication, i.e. GJCs and paracrine communication, may promote and expand radiation-induced biological effects in *in vitro* and *in vivo* settings [15–19]. Although the occurrence of a wide diversity of radiation-induced bystander effects (RIBE) (e.g., apoptosis, DNA damage, chromosome aberrations, changes in proliferation and differentiation, oncogenic transformation) has been well documented for more than two decades, researchers are still groping in the dark when it comes to the molecular spreading mechanism involved. We here review current knowledge of the role of Cx channels in radiation-induced effects and specifically address the molecular mechanisms that may underlie Cx-mediated effects with a focus on oxidative stress and IP_3/Ca^{2+} signaling. By broaching the tight interplay between Cxs, oxidative stress and IP_3/Ca^{2+} signaling, we provide a conceptual framework for intercellular Ca^{2+} signaling as a self-perpetuating mechanism that dictates the RIBE.

2. Setting the scene: concepts of connexin channels, Ca^{2+} signaling and bystander effects

Cxs are tetraspan proteins that contain four transmembrane domains, two extracellular loops, one cytoplasmic loop, and one C-terminal and N-terminal tail. At present, 21 human Cx proteins have been identified. Their nomenclature is based on their molecular weight that ranges from 25 to 62 kDa. Cxs are present in most organs and display a tissue/cellular specificity with Cx43 being the most abundant and wide-spread Cx species in mammals [20,21]. Cx proteins are best known in the scientific community as the building stones of GJCs, i.e. cell-to-cell channels that serve as passageways between the interiors of neighboring cells [11]. They are formed by the docking of two hemichannels that belong to the membrane of adjacent cells and are composed of six Cx subunits. Depending on their subunit composition, they are characterized by specific unitary conductances, permeability profiles and regulatory properties [22]. Most cells of healthy tissue, except for differentiated skeletal muscle cells and circulating blood cells communicate through GJCs [11,23]. These channels nurture diverse functions within all tissues by allowing the direct exchange of ions and low molecular weight molecules (<1.5 kDa) including glucose, glutamate, glutathione, cyclic adenosine monophosphate (cAMP), adenosine 5′-triphosphate (ATP), IP_3 and ions (e.g., Ca^{2+}, K^+, Na^+, Cl^-) between the cytoplasm of neighboring cells [11,24]. As such, gap junctional intercellular communication is considered a key mechanism for synchronizing physiological functions of cells and for the maintenance of tissue homeostasis. The picture is, however, not as straight forward as functional Cx channels are also present in a hemichannel configuration in non-contacting plasma membranes and provide an additional pathway for communication. Indeed, these channels allow for the exchange of ions (Ca^{2+}, Na^+) and low-molecular weight substances (nicotinamide adenine dinucleotide, ATP, glutamate, glutathione, prostaglandin E2 (PGE2) and IP_3) between the intra- and extracellular environment, thus ministering autocrine and paracrine signaling [10]. Moreover, another family of transmembrane proteins, called the pannexins (Panx 1–3), can form channels with a similar topology and function as Cx hemichannels. To prevent misconceptions, these channels will be denoted as Panx channels throughout the review [25,26].

Although functional hemichannels have been identified more than two decades ago, the biological significance of these channels is still a matter of debate. It is difficult to understand how a poorly selective plasma membrane channel, with a conductance about twice the conductance of the corresponding GJC (e.g., ~220 pS for Cx43 hemichannels [27]), can contribute to the normal functioning of a cell. It is therefore advocated that hemichannels remain closed under most conditions to prevent leakage of metabolites and nutrients, and loss or entry of ions that would otherwise lead to cell dysfunction and cell death [12,28]. Under resting conditions, Cx43 hemichannels indeed exhibit a low open probability in cultured cells [27,29], but it can be increased upon exposure to various, mostly stress-associated stimuli such as ischemic or pro-inflammatory conditions [30,31], oxidative stress [32] or an increase in $[Ca^{2+}]_i$ [33]. Using an *in vitro* model of localized apoptosis induction, we previously demonstrated that both Cx43 GJCs and hemichannels provide intercellular passageways for the spread of apoptosis between glioma brain tumor cells transfected with Cx43 [13]. Bystander apoptosis was detected in regions located as far as several 100 μm away (detection limit) from a zone that was exposed to the apoptotic stimulus cytochrome C. GJCs are anticipated to accommodate the direct exchange of pro-apoptotic signals between cells, while hemichannels can release substances that act in a paracrine manner on surrounding cells thereby modulating the spatial spreading of cell death [12,13]. Experimental data furthermore pinpointed the involvement of $[Ca^{2+}]_i$ increases in the induction of apoptosis mediated by both Cx43 channel types [13].

Ca^{2+} is a versatile and ubiquitous cellular signal. Variations in $[Ca^{2+}]_i$ control a multitude of biological processes over a wide temporal range through the activation of or interaction with various signaling pathways [34]. The concentration of free Ca^{2+} in the cytoplasm is kept to a minimum (~100 nM), while it reaches higher concentrations (μM to mM levels) at the extracellular environment and in intracellular organelles that serve as intracellular Ca^{2+} stores. To ensure specificity, Ca^{2+} signals are highly organized in time and space due to the action of buffers, pumps and channels/exchangers that are present in the cytoplasm, plasma membrane or in internal stores [35–37]. Increases in $[Ca^{2+}]_i$ can be rapidly induced either by Ca^{2+}-influx *via* channels in the plasma membrane or by mobilization from the intracellular stores. The endoplasmic reticulum (ER) is the largest and most controlled intracellular Ca^{2+} store. Ca^{2+} release from the ER can be mediated by the phospholipase C (PLC)-mediated generation of the second messenger IP_3 and its subsequent binding to its receptor, i.e. the IP_3 receptor (IP_3R), that is located on the ER surface (Box 1) [38]. Mitochondria furthermore play an important role in shaping Ca^{2+} signals by taking up Ca^{2+} that is released from the ER [39,40]. As a result, rapid and highly localized cytosolic Ca^{2+} spikes may co-occur or evolve into slower global $[Ca^{2+}]_i$ changes that are either transient, repetitive or sustained. Increases in $[Ca^{2+}]_i$ are not always restricted to the cytosol of a single cell, but can be propagated to vicinal cells. The mechanism is primarily based on the diffusion of IP_3 through GJCs and the release of ATP as a

Please cite this article as: E. Decrock, et al., Calcium, oxidative stress and connexin channels, a harmonious orchestra directing the response to radiotherapy treatment? Biochim. Biophys. Acta (2017), http://dx.doi.org/10.1016/j.bbamcr.2017.02.007