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Opportunities and challenges of radiotherapy for treating cancer

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Abstract

The past 20 years have seen dramatic changes in the delivery of radiation therapy, but the impact of radiobiology on the clinic has been far less substantial. A major consideration in the use of radiotherapy has been on how best to exploit differences between the tumour and host tissue characteristics, which in the past has been achieved empirically by radiation-dose fractionation. New advances are uncovering some of the mechanistic processes that underlie this success story. In this Review, we focus on how these processes might be targeted to improve the outcome of radiotherapy at the individual patient level. This approach would seem a more productive avenue of treatment than simply trying to increase the radiation dose delivered to the tumour.

Introduction

Radiation therapy is the most-effective cytotoxic therapy available for the treatment of localized solid cancers. The success of this approach is exemplified by the fact that about 60% of patients with cancer in the USA continue to receive curative radiation therapy—a century after its invention and despite advances in many other treatment modalities. In the past 20 years, there has been a dramatic increase in physical dose delivery options in clinical radiation therapy.[1] Major improvements in computer-aided, 3D treatment-planning systems with high-precision techno logy that include tracking organ motion during delivery have been noted. These delivery improvements have been aligned with advances in imaging that support strategies based on intensity-modulated radiation therapy (IMRT), whereby specified doses can be targeted to avoid critical structures and to falloff sharply outside the cancer volume, thereby minimizing dose and toxicity to neighbouring normal tissue. The armamentarium available to clinicians to achieve the same aim has been expanded by novel accelerators for the delivery of proton and heavy-ion charged-particle therapy (CPT) that enable a sharp increase in dose at a very defined depth (Bragg peak),[2] albeit at greatly increased monetary cost. In some situations, these new approaches enable higher fraction sizes to be delivered, with desirable decreases in treatment times, as in stereotactic (body) radiation therapy (SBRT).[3] Demonstrating that the advances in physical delivery of ionizing radiation have translated into therapeutic benefit, however, has been challenging.

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The implementation of these new technologies has been largely empirical and driven by the belief that increasing dose will increase cure, rather than being guided by solid clinical or radiobiological data. We present the view that ionizing radiation is rather different from other cancer therapies, and that biological and chemical targeting should take account of these differences and be optimally integrated with new radiation-delivery techniques. This approach is more likely to advance radiation therapy than strategies aimed at increasing the radiation dose delivered to the tumour.

Classic radiobiology phenomena

For the past 40 years, radiation oncologists have often been guided by the classic radiobiological phenomena that underline fractionated radiotherapy and that are enshrined by Withers as the '4Rs of radiotherapy'—namely, repair, redistribution/reassortment, repopulation, and reoxygenation.[4] These four phenomena are often extended by a fifth 'R', that of intrinsic radiosensitivity, defined as the initial DNA damage caused by radiation.[5] These phenomena help explain how conventional daily low-dose fractions of around 2 Gy, given five times per week, can exploit differences between normal tissues and tumours, and yield outcomes that are often superior to radiotherapy given in fewer single large doses.[4] These principles have stood the test of time; however, they come from an era before advances such as IMRT and molecular profiling were available, and their adaptation to new clinical realities has been slow.[6] Even treatment failure in the form of radioresistance, which is known to be associated with certain histologies (such as melanoma and glioblastoma) or oncogenic mutations (for example, *KRAS* and *EGFR*), or tumour-associated hypoxia and their relationship with radiotherapy dose delivery requires further elucidation. Nevertheless, the magnitude of these biological influences on outcome argues that the widely held belief of increasing the radiation dose, even if practically achievable with new technologies, is unlikely to significantly increase the cure rate in many cancers. Rather, a paradigm shift towards biological interventions that are tailored specifically to radiation-related parameters is needed.[7,8]

Recent discoveries in cancer research have given some hope for new avenues of radiation therapy. First, mutations in DNA-damage-response (DDR) pathways have been found to occur very frequently in cancer. These mutations can promote radioresistance, genomic instability, and increase tumour heterogeneity, but they might also represent a potential Achilles' heel for intervention in cancer radiation therapy and in immunotherapy.[7,9] Second, novel biologically targeted agents have been introduced, which, although not necessarily designed for interaction with radiation, might radiosensitize tumours. Even if their synergy with radiation is limited, such agents often have little cytotoxic action when used alone; thus, radiotherapy might be needed to achieve a sufficient cell kill. Furthermore, many examples exist in which a subset of cells escapes the attention of such agents by virtue of losing the targeted molecule or as a result of bypass (escape) pathways. In such scenarios, targeting radiation therapy to the residual tumour deposits would seem appropriate. Third, radiation therapy can alter the tumour microenvironment, which argues for its role as part of a combination therapy; for example, to engage the immune system or improve drug penetration. Hypofractionated SBRT protocols might be superior in this regard.[10] Fourth, the ability of IMRT and CPT to deliver radiation dose more precisely to tumour sites will help

to localize and amplify any drug–radiotherapy synergy, which would be important for drugs that target radiation-related processes such as DNA repair.[7]

Of note, radiation therapy is unique in that the normal tissue adjacent to the tumour will often receive doses that are close to the maximum doses that can be tolerated. Addition of even a minimally cytotoxic drug could, therefore, be sufficient to precipitate a crisis. Ironically, radiation dose de-escalation might, on occasion, be the only way to increase the therapeutic benefit of some combined therapies,[11] which is a clinically challenging concept. We believe that many potential biological approaches can increase the radiotherapeutic benefit, but we focus herein on only a few that we consider the most promising.

Targeting DNA damage and repair

Ionizing radiation is unique as an anticancer modality in its ability to generate lethal lesions. Clusters of ionization events are generated ubiquitously in cells and tissues, which in turn cause clusters of diverse molecular lesions. In DNA, simple forms of damage can be repaired with relative ease, but dense lesions formed within one to two loops of the helix are more problematic, with complex DNA double-strand breaks (DSBs) being formed that are often lethal to cells. DSBs can be formed as a direct result of clusters of base changes and strand breaks or indirectly during lesion processing and repair, or by conversion at replication forks through homologous recombination (HR).[12] In any event, this proclivity of ionizing radiation to form large, complex DSBs explains its efficiency as a cytotoxic agent. DNA-repair pathways are, therefore, valid targets for radiotherapeutic interventions in cancer therapy.[7] What is perhaps surprising is the growing evidence for interconnectivity between the diverse mechanisms underlying the ‘5Rs of radiotherapy’. For instance, radiosensitization resulting from ‘targeting’ one pathway could in fact be a result of unexpected effects on DNA repair.

This crosstalk between pathways in response to radiation exposure has been obvious since the DDR was discovered.[13] This evolutionarily conserved signalling cascade senses and responds to DNA DSBs to regulate cell-cycle progression and cell-fate decisions, such as apoptosis and senescence, with the main aim of maintaining genomic integrity. Investigations into the crosstalk were greatly facilitated by an improved assay for DSBs.[14] The protein product of the gene mutated in ataxia telangiectasia (*ATM*), along with ATR (ATM-related and RAD3-related) and DNA-PKcs (DNA-dependent protein kinase catalytic subunit) form a phalanx of kinases triggered by DNA damage. One result is that ATM phosphorylates serine139 of a histone variant, H2AX, in the surrounding chromatin to produce γH2AX, which marks the DSB site. The number of γH2AX radiation-induced foci (RIF) in the nucleus is now used routinely to assess the amount of DNA damage and its repair kinetics. This RIF assay has been extended to interrogate other proteins involved in the dynamic orchestration of chromatin-directed DNA-repair programmes.[15–17] The nature of the molecules within the RIF reflects the repair mechanism involved. Each repair mechanism is associated with an exclusive set of recruited RIF proteins, but many molecules that include H2AX, ATM, MRN, BRCA1, PARP-1, and DNA-PKcs, are involved in more than one mechanism, illustrating the considerable crosstalk between DNA-repair systems.

Non-homologous end joining (NHEJ) is the main DNA DSB-repair mechanism evoked after ionizing radiation exposure. This pathway normally requires DNA-PK activation, but can also proceed by a slower, alternative DNA-PK-independent pathway (error-prone microhomology-mediated end joining). NHEJ catalyses a simple rejoining of two DNA DSB ends irrespective of their origin[18,19] without or with only minimal (for the alternative pathway) guidance from a template and, as a result, is an error-prone process. By contrast, the other major DSB-repair system, HR, faithfully restores the DNA sequence using the sister chromatid as a template,[20] and is therefore active only in late S and G2 phases of the cell cycle (Figure 1).[21] ATR responds to a wider array of DNA damage than ATM, and is activated through DSB resection and the ssDNA-replication protein A (RPA) complex, and responds to stalled or collapsed replication forks.[22] Defects in HR precipitate increases in mutational load, tumour heterogeneity and cancer progression, indicating the critical role for HR in maintenance of genomic integrity as well as DNA repair.

Initial DNA damage is dependent on the nature (or quality) of the ionizing radiation, which defines the spatial density of the ionization events. For example, densely ionizing heavy-ion CPT has a high relative biological effectiveness. That the quality of ionizing radiation can also affect the balance between repair pathways has now become clear,[23,24] with heavy ions causing a shift towards greater use of DSB resection and alternative-end joining in G1, as well as HR in late S/G2, when compared with sparsely ionizing photon irradiation.[25] Clearly, the time that tumour cells spend in G0/G1, overall, will impact on the balance between repair mechanisms,[26] although much remains to be learnt about this aspect of the response. For example, the exact molecular mechanisms defining this balance and the default pathway chosen require clarification; however, findings that low-dose pre-exposure chromatin structure,[27] and cell-cycle phase can influence the balance between the repair pathways suggest considerable complexity in the DNA-repair process.

Variation in radiosensitivity with cell-cycle phase (Figure 1), with cells in late S phase being markedly more resistant than those in G1, is a presumed result of the high efficiency of HR and changes in chromatin organization. This phenomenon is seen in another radiobiological tenet, namely reassortment, which enables fractionation to preferentially kill proliferating cells within radiosensitive cell-cycle phases. The link between HR and the S/G2 cell-cycle phase[28,29] has been attributed largely to a requirement for cyclin-dependent kinase 1 (CDK1) for DNA resection (Figure 1).[30] CDK1 activates HR by phosphorylating key recombination factors, and phosphorylates the XRCC4-like factor (XLF; also known as Cernunnos) to downregulate NHEJ, at least in yeast.[31] Repair is also linked to the cell cycle by Chk1, an effector kinase of ATR that promotes checkpoint arrest in S and G2/M, and DNA repair through RAD51 and HR.[32] Chk1 inhibition leads to DNA damage and cell death,[33] and is a potential target for improving the outcome of radiation therapy.

Of note, many chemotherapeutic drugs that are used in conjunction with radiotherapy target or interfere with DNA repair. The nucleoside analogues 5-fluorouracil (5-FU) and gemcitabine, and the topoisomerase I inhibitors primarily target HR, whereas cisplatin plus radiation synergize by impairing the NHEJ pathway.[7,34] Despite clear treatment success, the reality of chemoradiation in many clinical contexts is, however, that it often comes with increased toxicity and only a modest improvement in outcome,[11] which emphasizes the need

for more in-depth consideration of how best to translate what we know about DNA repair into an increased therapeutic benefit.

Most cancers have defects in DNA repair, but little is known about the impact of such mutations on outcomes in radiation therapy. The current 'poster child' for targeting DNA repair in the clinic is poly(ADP-ribose) polymerase-1 (PARP-1). PARP-1 and its 17 related family members have central roles in many cellular processes. This protein is a sensor of SSBs in DNA, but has a plurality of functions in DNA replication, transcription, DSB repair, histone/chromatin modification, and cell death, as well as inflammation.[35] The central concept driving the use of PARP inhibitors is that they block SSB repair, and increase the number and complexity of lesions that have to be dealt with by HR (Figure 2). This concept that radiation- induced, non-DSB, clustered DNA-damage repair impacts on HR, is further supported by the S-phase specificity of killing by PARP inhibitors.[36] Defects in HR repair, which might be caused by a *BRCA* mutation, result in double jeopardy—analogous to 'synthetic lethality' (Figure 2). Initially, this explanation was proposed for the single-agent efficacy of PARP inhibitors in *BRCA*-mutated breast cancers.[37,38] However, many DNA-repair mutations other than *BRCA1* might sensitize tumours to PARP inhibitors, which could potentially explain the relatively high response rate in patients with non-*BRCA*-mutated ovarian cancer.[37] Because radiation therapy itself exploits differences in repair capacity between tissues, that the combination of radiation with PARP inhibitors is effective against cancers that have BRCA, MRE11, and other DNA-repair-protein mutations is no surprise.[39–44] However, PARP-1 is also activated by oxidative and nitrative stress, and is a known cofactor for nuclear factor κB (NF-κB)-driven inflammatory gene expression;[45] radiosensitization by PARP inhibitors might also be the result of inhibition of this crucial survival pathway (Figure 2).[46] Not surprisingly, many clinical trials of PARP inhibitors in combination with radiation therapy are ongoing.[41,47] In evaluating their outcomes, it must be remembered that patients with germline mutations in DNA-repair genes will be also at increased risk of radiation-induced normal tissue toxicity in the presence of PARP inhibitors, and that different PARP inhibitors are available with differing specificities for what is a functionally very-complex family of molecules. A salutary lesson comes from the failure of the putative PARP inhibitor iniparib, in combination with gemcitabine/carboplatin, in a phase III clinical trial in women with triple-negative breast cancer:[48] iniparib was later shown to have little PARP-specific activity even in cells *in vitro*.[49]

Many new drugs have been developed that target molecules involved in DNA repair and the DDR, including ATM, ATR, Chk1/2, DNA-PKcs, and WEE1, and other targets specific to HR.[50] Some of these are in phase I clinical trials. There is hope for further rational approaches to combined drug–radiotherapy treatment, although their optimal implementation will probably require prospective molecular profiling to define the tumour-related DNA-repair and DDR processes.

Chromatin structure and targeting

One incidental consequence of the discovery of RIF as markers for DNA DSBs was that it illuminated the spatiotemporal organization of repair processes within functional and structural chromatin domains. Increasing evidence indicates that chromatin architecture

imposes important constraints on DNA damage and repair, and that the chromatin arrangement changes on formation of DSBs. Chromatin structure is an e merging potential target for radiation therapy.

Much of the damage from photon ionizing radiation is indirect, through the generation of free radicals, especially reactive oxygen species (ROS) that are generated by radiolysis of water. Radiation with densely ionizing tracks, such as heavy-ion CPT, is less dependent on this pathway. At the molecular level, little is known about how the tightness of the binding of chromatin-associated water, histones, antioxidants, and other molecules influences the lesions formed by ionizing radiation, but some evidence from isolated nuclei suggests that ROS cannot easily penetrate condensed chromatin.[51] A role for chromatin in determining radiosensitivity was suggested more than 25 years ago,[52] but RIF assays have enabled more detailed investigations of differences in DNA damage and repair between condensed heterochromatin and looser, more transcriptionally active euchromatin. RIF are not readily detected in the centre of hetero chromatic regions following ionizing radiation, but damaged DNA seems to relocate to the periphery of these regions where ATM-dependent repair occurs,[53–55] which slows the repair process compared with DNA damage that occurs in euchromatin.[56–58] These differences have been found with the use of both sparse and dense ionizing radiation, and have led to the conclusion that chromatin and nuclear architecture influences the dynamics and extent of DNA damage and repair even within one cell-cycle phase. Extrapolating these data to cell survival needs caution, but several reports suggest that radioresistant cells have higher heterochromatin levels than radiosensitive cells.[54,59] Also, chromatin structure varies with cell activity and status, and this variation is a suggested explanation for observed differences in radiosensitivity between T-cell subsets.[60] In the longer term, greater understanding of the dynamics of RIF viz-a-viz chromatin structure should give rise to new biomarkers of radiosensitivity and identify novel therapeutic targets.

Little is known about how the ionizing-radiation-induced local and global chromatin unwinding processes are regulated following lesion formation, and during ongoing DNA repair and restructuring, so as to maintain genomic integrity.[61] Radiation-induced local decondensation of DNA in the vicinity of DSBs could potentially enhance the mobility of damaged chromatin domains and increase repair fidelity. Remarkably, additional roles for ATM and its effector kinase Chk2 have been identified in relaxing the heterochromatin structure to allow access of the DNA-repair m achinery.[62–64] This involves phosphorylation of transcription intermediary factor 1-β (TIF1-β; also known as TRIM28 and KAP1), a master repressor that maintains local heterochromatin architecture.[65,66] A similar chromatin-modifying role has been suggested for tumour suppressor p53-binding protein 1 (53BP1).[67] The forces that operate to compact or relax chromatin and those that tether it to nuclear structures must be carefully controlled to promote different aspects of repair in heterochromatin and euchromatin, and the slow ATM-dependent DSB repair of densely packed chromatin domains at the periphery referred to earlier is probably just one manifestation of these forces at work. Radiation therapy clearly disrupts local and distant chromatin architecture, its epigenetic landscape, and gene function. How radiotherapy impacts on the complex social interactions between multiple chromatin regulatory factors remains unclear, but the evidence is growing that higher-order chromatin structure can

dramatically affect the radiation responses, and provide targets for improving the benefit of radiation therapy.

The primary mechanisms that dictate chromatin dynamics are methylation and histone acetylation. Both potentially alter radiation responses, with agents targeting the latter process being more advanced in clinical testing.[68] Several inhibitors of histone deacetylases (HDACs) have entered clinical trials.[69] Vorinostat (SAHA) and romidepsin (FK228) were shown to improve outcomes for patients with cutaneous T-cell lymphoma, and others HDAC inhibitors are being tested in a variety of cancers.[70] Combining radiation with HDAC inhibitors was demonstrated to be a promising approach by findings that these drugs radiosensitize various cancer cell lines in culture and *in vivo*.[71–77] This combination approach was first tested in the clinic in 2009,[78] and since then several trials have been initiated and have produced some promising early data.[79] The main rationale presented for the use of these inhibitors is that they prevent HDACs from maintaining tightly packed chromatin that might promote radioresistance.[59,80] However, numerous non-histone proteins are deacetylated by HDACs,[68,80] which presents many other possible mechanistic interpretations for the anticancer action of HDAC inhibitors. Similarly, the existence of four classes of 18 different HDACs, all with multifaceted roles in coordinating intracellular signalling pathways with genetic and epigenetic functions, supports alternative mechanisms. Not surprisingly, multiple end points for HDAC-inhibitor activity have been reported, including gene expression, cell-cycle arrest, cell differentiation, antiangiogenesis, cell death, and autophagy.[81] In addition, HDAC inhibitors have been reported to generate ROS and modulate redox levels in cells,[82–87] resulting in DNA damage and activation of the DDR.[88,89] Others have shown that key DNA-repair molecules including Ku70/Ku80, DNA-PK, RAD50, RAD51, BRCA1/2, and MRE11 are downregulated by vorinostat and other HDAC inhibitors in combination with radiotherapy, leading to RIF persistence.[75,77,90–92] Furthermore, DNA-repair-defective cancers are more sensitive to HDAC inhibition.[69] For many reasons, these drugs might make good candidates for combination with radiotherapy, but the mechanisms might be obscure. Importantly, reports suggest that HDAC inhibitors do not radiosensitize normal cells in the same way as they sensitize cancer cells.[74] In fact, HDAC inhibitors have even been shown to protect against the lethality of whole-body irradiation in mice,[75,93] indicating a possible radiotherapeutic differential. This radioprotection might be due to their anti-inflammatory effects, evidence of which is growing.[94–96] A critical issue for further advancement of HDAC inhibitors is their specificity and validation. Currently, two opposite design strategies are underway: highly selective and/or multitargeted. It will be interesting to see which of these approaches is most effective in a radiation-therapy setting.

Growth factors and radiotherapy

Recently, one of the most interesting convergences in radiobiology has taken place between growth factor signalling and DNA repair. This relationship is exemplified by EGFR, which has become a paradigm for growth-factor-driven radioresistance.[97–99] EGFR is overexpressed or mutated in intestinal, lung, brain, and head and neck cancers, among other tumours, and this pathway has been targeted using monoclonal antibodies, such as cetuximab, and small-molecule inhibitors, including gefitinib and erlotinib.[100] Monotherapy

with these agents is not particularly effective, in part because of bypass mutations or pathways, which spurred efforts to optimize the combination of these agents with radiotherapy and chemotherapy. Building on earlier promising results, a phase III clinical trial with radiotherapy in locoregional advanced-stage head and neck cancer showed that cetuximab markedly increased survival from 29 months with radiotherapy alone to 49 months, with a 9.2% overall long-term survival.[101,102] Many other phase II/III trials that are combining radiation with cetuximab are currently underway in various treatment settings.[97] Of note, cetuximab failed to improve outcomes when added to a radiotherapy–cisplatin regimen, [103] suggesting convergent pathways. Also, in rectal cancer combining cetuximab with chemoradiation produced disappointing results, possibly because the antiproliferative effect of cetuximab compromised the activity of the chemotherapeutics.[104] One potential mechanism of radiosensitization by EGFR inhibition that has been studied is through p53-dependent G1 arrest, but this pathway does not necessarily lead to improved tumour control.[105] These findings provide a salutary lesson that unless the mechanistic basis for radiosensitization is known and biomarkers are available, success in one system might not be easily translated to another.

The initial rationale for combining EGFR inhibition with radiotherapy was that ionizing radiation activates multiple tyrosine-kinase receptors and signal-transduction pathways,[106] including PI3K/AKT and RAS/RAF/MEK/ERK, and would therefore drive cells to favour increased survival and proliferation.[98,107] Radiation-driven activation of EGFR is a rapid, ROS-dependent process involving phosphatase inactivation.[108,109] An essential feature of growth-factor signalling, also involving ROS, is the shuttling of activated protein kinases between the cytoplasm and nucleus.[110–112] In fact, nuclear EGFR in tumours has been linked to a worse prognosis.[113] The Rodemann group[114,115] were first to present another explanation for EGFR-driven radioresistance by linking ionizing radiation- induced EGFR nuclear translocation to superior DNA repair, and demonstrating that nuclear EGFR enhanced DNA-PKcs activity. EGFR has also been reported to bind to excision repair cross complementation group 1 (ERCC1) protein[116] and EGFR-stimulated PI3K/AKT to interact with DNA-PKcs.[117] Furthermore, somatic activating mutations in *EGFR* that have been linked to gefitinib and erlotinib responsiveness in patients with non-small-cell lung cancer, have been reported to make cells more radiosensitive than those with non-mutated *EGFR*.[117] These effects extend to chromatin structure, as EGFR can be found in RIF, where it associates with histone acetyltransferase KAT5 (Tip60) to regulate ATM phosphorylation of TIF1-β with resultant heterochromatin relaxation.[118–120] One message from these studies is that cells can integrate cues from the microenvironment into DNA repair and chromatin dynamics to ultimately influence cell death and survival. This level of integration will probably also exist for other signalling pathways, which might expand the possibilities for targeted radiotherapeutic intervention. Unfortunately, the complexity of the interactive network governed by growth factors and/or their receptors, such as EGFR, makes it difficult to develop biomarkers that could reliably predict outcome, and clearly many factors need to be accounted for and optimized before this approach can be reliably used in the clinic.

Cancer stem cells and radiotherapy

Major concerns in cancer therapy relate to the role of cancer stem cells (CSCs), and whether these cells express the same targets as the cancer as a whole and have the same sensitivity to cytotoxic agents. The CSC hypothesis posits a relatively small number of stem cells as a self-renewing force that drives tumour growth and metastasis, and that CSCs contribute disproportionately to tumour recurrence.[121,122] CSCs from several human solid cancers seem to be particularly resistant to radio therapy,[121,123–128] although reports to the contrary exist.[129,130] Radioresistance has been associated with a metabolically quiescent state,[131] increased levels of free-radical scavengers, lower ROS levels, increased DNA repair, cell-cycle checkpoints,[121,124,132] and survival.[133,134] The finding that CSCs are not resistant to heavy-ion CPT, which causes more direct clustered and complex DNA damage, also suggests that indirect free radical, ROS-directed pathways are involved in their resistance to photon radiation.[135] Chromatin in CSCs has been reported to be more condensed than in non-CSCs,[59,136] and this factor might also have a role in radio resistance, although this might oversimplify these complex dynamic systems.

Under conditions of pathological stress, including radiation,[137,138] hypoxia,[139] and oncogene expression,[140] checkpoints that restrict developmental cellular outputs can be lifted, allowing cells to reprogramme for ‘stemness’.[141] Such reprogramming could be an essential part of normal healing, but during fractionated courses of radiation it could generate a nidus for recurrence. Reprogramming can be likened to induction of pluripotency in somatic-cell populations via the four Yamanaka factors (OCT3/4, SOX2, KLF4, c-MYC) that are highly expressed in embryonic stem cells, which demonstrates the plasticity inherent in many cell types.[137] This process also highlights the substanstial phenotypic and functional heterogeneity within CSCs that manifests as a continuum of stemness-related gene expression.[137,142] This heterogeneity might explain why definitive CSC markers are lacking, although major characteristic pheno types are recognized. One could argue that radioresistance and reprogramming of CSCs add to the classic 4Rs and 5Rs that impact the outcome of a course of fractionated radiotherapy, making a case for 6Rs.

Heterogeneity within CSC populations also makes a ful understanding of the effects of therapies challenging. Reports indicate that PARP1 is overexpressed in certain CSC subsets and that these cell populations are more sensitive to PARP inhibitors.[143,144] HDAC inhibitors have been reported to radiosensitize CSCs.[59] By contrast, EGFR-targeting agents might be less efficacious when CSCs lack expression of EGFR, as has been suggested for some CSCs from head and neck cancers.[145] Exclusive targeting of CSCs to reduce radioresistance and to block stress-induced reprogramming remains in its infancy, but therapeutic avenues for elimination of CSCs have been identified within the developmental pathways driven by the four key stem-cell factors that include fibroblast growth factor (FGF)/MAPK, Notch, WNT, Hedgehog (HH), JAK/STAT, and transforming growth factor β (TGF-β) pathways.[146,147] Not surprisingly, these signalling cascades are often dysregulated in cancer. Some of the first stem-cell-targeting agents tested were γ-secretase inhibitors (GSIs) that disrupt Notch signalling, and were found to radiosensitize tumours in preclinical studies.[148–150] Some GSIs are in clinical trials for cancer treatment,[151] although global inhibition of γ-secretases is associated with toxicity.[152] Interestingly, chloroquine, which

can radiosensitize tumours[153–155] and unmask radiation-induced antitumour immunity,[156] showed some specificity for CSCs that was mediated by inhibition of CXCL12/CXCR4 and the HH pathways, rather than by blocking autophagy—the activity generally attributed to this agent.[157]

A commonly expressed CSC marker is CD44, in particular, the splice variant CD44v. That CD44v is associated with radioresistance in prostate cancer cells might not be coincidental.[158] An intriguing aspect of CD44v-positive CSCs is that they regulate their ROS levels through the activity of the cysteine transporter subunit xCT, a subunit of the cysteine–glutamate antiporter system that promotes glutathione synthesis.[145,159] xCT is upregulated in about 30% of triple-negative breast cancer cell lines[160,161] and might represent a good target for radiosensitization.[162] Interestingly, the HDAC inhibitor vorinostat has been shown to normalize xCT-containing transporter levels in gliomas with an accompanying increase in ROS levels. [86] The cysteine transport system maintains intracellular cysteine and glutathione pools in many cells to counter oxidative stress. This system is under control of nuclear factor-erythroid 2 p45-related factor 2 (Nrf2), which provides a potential link for CSC radioresistance through expression of free -radical scavengers.

The transcriptional factor Nrf2 is the master regulator of cellular redox homeostasis and cytoprotection, acting by upregulating a plethora of antioxidant response element (ARE)-bearing gene products, including γ-glutamylcysteine synthetase (GCS), the limiting enzyme of glutathione synthesis (Figure 3). This battery of antioxidative and cytoprotective gene products generally promotes cell survival and cellular radioresistance.[163] As oxidative stress is a hallmark of most cancers, not surprisingly, Nrf2 or its inhibitor Keap 1 is frequently mutated, for example, in lung cancer.[164,165] Intriguing evidence indicates that oncogenic *KRAS* confers tumour chemoresistance by upregulating Nrf2.[166] By regulating redox, the Nrf2 network also guides anti-inflammatory responses and counterbalances proinflammatory transcription factors, such as NF-κB, setting the T-helper cell type 1/2 (T_H1/T_H2) immune balance.[167] Moreover, the Nrf2 network has metabolic influences, facilitating flux through the pentose phosphate pathway, increasing NADPH regeneration and purine biosynthesis, and seems to direct metabolic reprogramming during cellular stress.[168]

Redox processes are central to the basic functioning of cells and tissues, as well as to radiation damage.[169] They are key to regulating biochemical pathways and networks operating to affect signal transduction, DNA and RNA synthesis, protein synthesis, enzyme activation, metabolism, and regulation of the cell cycle. The level of ROS generated and the initial redox state are important elements in deciding responses. Quantitative assessment of these factors is difficult, especially because of intracellular compartmentalization, although new and better probes are becoming available.[169,170] The redoxome is large and many important transcriptional programmes, including those driven by Nrf2, NF-κB, and AP-1, can be triggered by redox changes, and by ionizing radiation. ATM can be directly activated by oxidation by a mechanism distinct from the DDR,[171,172] as can AMP-activated protein kinase,[173] and many phosphatases. The largely proinflammatory effects of ionizing radiation are through the generation of free radicals and activation of NF-κB.[174] Redox is, therefore,

a natural, well-recognized target for radiation response modification. Importantly, marked differences in redox status exist between normal and cancer cells, although exploiting these variations remains in its infancy. Metformin, a drug widely used to treat type 2 diabetes, regulates redox, has direct anti-CSC activity,[175] and is a radiosensitizer.[176] Clinical trials of metformin combined with radiation show promising results,[177–179] but the increased acute locoregional toxicity of radio therapy observed in diabetic patients receiving metformin urges caution and detailed consideration of the therapeutic ratio.[180]

Persistent damage, ROS, and senescence

Classic radiobiology concepts posit that ROS are generated very rapidly DNA damage and repair that is measured in minutes to hours, whereas time to expression of tissue damage is dictated by its turnover time. These contentions are only partly true. In fact, ionizing radiation can trigger waves of self-inflicted oxidative stress with DNA and tissue damage that can persist for weeks and months after exposure.[134,163,181–183] These effects are associated with inflammatory cytokine production.[174] Senescent cells can be detected *in vitro* by a hallmark increase in $p16^{INK4a}$, $p21^{CIP1}$, and β-galactosidase expression.[184] *In vivo*, irradiation of mice and human cells results in similar $p16^{INK4a}$ and DNA-damage foci that persist for many months.[183] Senescent cells remain metabolically active and undergo a plethora of changes, including the development of a senescence-associated secretory phenotype (SASP) with persistent nuclear foci and elevated Chk2 and p53 levels as part of a delayed DDR.[185] DNA lesions that persist have been called ‘DNA segments with chromatin alterations reinforcing senescence’ (DNA-SCARS) and shown to functionally regulate multiple aspects of the senescent phenotype.[185] A major feature of SASP is secretion of inflammatory cytokines, especially IL-6, IL-8, growth factors, and proteases in response to DNA damage.[186,187] Chronic proinflammatory cytokine responses involving recurring spikes of tumour necrosis factor α (TNF-α) and IL-1 have also been observed in tissues long after irradiation.[174] The relationship between these late manifestations of cytokine expression following radiation exposure requires further study, but they almost certainly contribute to the pathogenesis of radiation damage and might be instrumental in forming reprogramming niches. Juxtacrine, paracrine, and endocrine effects are an inevitable consequence of these responses that are often placed under the general rubric of radiation-induced, non-targeted, bystander effects.

The general concept that emerges is that after radiation exposure, acute tissue inflammation, which is largely pro-oxidant, can cause further DNA DSBs, and cell and tissue damage (Figure 3).[188] Under normal inflammatory circumstances, the danger of acute inflammation, which is required for pathogen removal, passes and in time a shift occurs to antioxidant processes with anti-inflammatory and growth-stimulatory cytokines that focus on tissue repair.[189] In this scenario, both cancer causation and prevention are possible outcomes of inflammation.[190,191] High doses of ionizing radiation disrupt this normal progression of events, generating chronic tissue damage that fails to heal appropriately; a concept supported by the recent development of mitigators of radiation damage that seem to target acute inflammation[192] and enhance stem-cell recovery after irradiation.

Local and systemic responses

As discussed, evidence indicates that radiotherapy can trigger a pro-oxidant, proinflammatory 'dangerous' microenvironment and that this microenvironment can serve as an immunological niche for the generation of adaptive immune responses.[174,189,193] Indeed, radiotherapy can generate tumour-specific immune responses both in mouse models and in humans.[194–196] Markers of activation include the upregulation of major histocompatibility complex and cell-adhesion molecules, expression of proinflammatory cytokine family members and their receptors, and molecules with damage-associated molecular patterns (DAMPS) for immune stimulation.[174] Antigen-presenting dendritic cells mature in this environment and gain in their ability to cross-present tumour antigens.[197] Optimal stimulation of adaptive immunity in radiation therapy might require threefold higher fraction sizes than the conventional 2Gy, which can be achieved by SBRT,[195,198] and perhaps by CPT. These higher doses may be needed to release sufficient neoantigens, DAMPS, and immunostimulatory molecules.[195,198] It is of interest that high individual doses might be suboptimal at stimulation.[195,198]

Success in immune-mediated tumour regression and in immunotherapy requires the tumour to express an appropriate neoantigen target landscape,[199] and investigations are ongoing to determine if radiotherapy can expand this landscape by promoting epitope spreading.[200] Release of highly charged histone and DNA fragments as well as oxidized molecules after radiotherapy might broaden immune responses, and genomic instability might, ironically, assist in doing the same. This possibility comes from evidence that microsatellite instability defines the immune microenvironment in some colorectal cancers,[201] that the presence of a high mutation rate improves immunotherapy responses,[9] and that suicide-gene therapy and photodynamic therapy increase tumour immunogenicity.[202,203] HDAC and PARP inhibitors as well as other chemo therapeutic agents could potentially enhance radiation-induced tumour immunity by similar mechanisms.

Obviously, radiotherapy does not always induce clinically relevant antitumour immunity, even in mouse models.[204] The tumour–host relationship is well established before therapy starts, often with an immunosuppressive tumour microenvironment to protect from excessive damage to self, although this varies between tumours.[205,206] Historically, the negative impact of the tumour microenvironment on radiotherapy outcomes has been ascribed to the presence of hypoxia, but this concept might be expanded considering the antioxidant effects of regulatory T (T_{REG}) cells, myeloid-derived suppressor cells (MDSC), M2 macrophages and anti-inflammatory cytokines, such as TGF-β, that can be associated with the tumour mass (Figure 3). An oversimplified view is that infiltrates are defined by tumour properties and fall between two polar extremes, best exemplified by plasticity within the myeloid lineage. For example, some tumours have functional M1 macro phages and are genuinely capable of generating T-cell-mediated immunity, even if this is often downregulated by suppressor cells, while others are polarized to an M2 phenotype early during growth and are generally ignored by the adaptive immune system.[205,207] A reductionist view is that these extremes most probably reflect the counterbalance between proinflammatory, pro-oxidant pathways that are regulated by factors such as NF-κB,[208,209] versus antioxidant, anti-inflammatory pathways that are regulated by factors such as Nrf2 (Figure 3). Whether

tumours with a proinflammatory immune profile have a better response to radiotherapy, which could be harnessed through the generation of adaptive immunity or through regulation of redox status, remains to be established.

Radiotherapy without additional intervention is unlikely to reproducibly overcome a well-established negative immune microenvironment present within many tumours, and might even enhance the immune suppressive environment. Thus, although radiation can act as an immune adjuvant, it can also enhance T_{REG}-cell representation, perhaps as a response to radiation-induced 'danger' signals,[196,210] and can also stimulate M2 macrophage infiltration.[211] The response probably varies from tumour to tumour but, at least in some preclinical models, radiation-induced vascular damage, which might be greater after SBRT or CPT,[10] can increase the extent of chronic, at the expense of acute, hypoxia.[211] Many tumours experience an influx of $CD11b^+$ myeloid cells that evolve into M2 growth-stimulatory macro phages in chronic hypoxic regions.[204,212–217] The radiation-induced influx of myeloid cells into these tumours can be prevented through the blockade of the HIF1α/SDF-1/CXCR4[214,218] or CSF1/CSF1R[213,216] axes resulting in tumour radiosensitization. Such approaches are now entering clinical trials.[219] In addition, angiogenesis is inhibited by such treatments, forcing irradiated tissues and tumours to become more reliant on vasculogenesis, which is a less effective process.[211,215,220]

Unmasking radiation-induced antitumour immunity by targeting negative immune forces has generated considerable enthusiasm. Not least because of the success of antibody-mediated inhibition of immune checkpoints that control antigen-specific T-cell responses against tumours. Triggering of the T-cell-receptor complex not only requires the antigen to be recognized on the surface of an antigen-presenting cell, but also needs a second signal to be sent in a coordinated fashion through a co-stimulatory receptor. The long-standing co-stimulatory proteins CD28 and B7, along with B7 and other protein families, are relevant targets for immune therapies. Some co-stimulatory protein are co-inhibitory (PD-1, PD-L1, CTLA-4, BTLA) rather than co-stimulatory (CD28, ICOS, 4–1BB, CD40, OX40, CD27) and operate to switch off responses.[221] These co-inhibitory molecules integrate with T_{REG} cells, MDSC, and M2 macrophages to downregulate immune responses. Tumours often thrive by expressing co-inhibitory molecules, but the remarkable efficacy of anti-CTLA4 and anti-PD-1/PD-L1 strategies in rebalancing antitumour immunity in favour of the host has excited the oncology community.[221]

Blockade of immune checkpoints enhances radiotherapy-induced immunity in preclinical models,[222–225] and clinical observations of out-of-field (abscopal) responses in patients receiving similar treatment have been noted,[226–228] leading to multiple ongoing clinical trials combining immune-checkpoint inhibitors with radiation therapy. In the longer term, full exploitation of this approach might be best achieved through a combination of radiation sensitizers and/or myeloid-cell inhibitors plus immune-checkpoint inhibitors, which raises the question as to how to interrogate the immune status of patients with a view to optimizing the choice of treatments. The duality within the host–cancer relationship is thought to arise from having to deal with pathogens without causing dangerous autoimmune responses, while healing tissues. As redox is a nexus for so many of the pathways that are intricately linked to cancer (oxidative stress, genomic instability, mutations, altered metabolism, the

tumour microenvironment, inflammation, and host immune responses), it will be interesting to investigate how redox-related or immune biomarkers reflect this status. This knowledge will be critical for evaluating how best to individually tailor therapies for combination with radiotherapy. Indeed, it would not be surprising if these markers also served as an index of response to classic radiation therapy.

Conclusions

In spite of the complexity of radiation responses, a unifying concept that has its roots in the polarizing effects of redox regulation in multiple pathways can integrate our understanding tumour radiosensitization and radioprotection of normal tissue. Multiple antioxidant and pro-oxidant pathways can be expressed, but a major direction for future advances in radiotherapy will be to examine how these pathways are balanced in patients with cancer, and how to tip this balance in favour of the host by therapeutic approaches, be they targeted to DNA repair, growth-factor inhibition, the tumour microenvironment, immune-checkpoint inhibition, CSCs, or mitigation and protection of normal tissue radiation damage.

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Key points

- Radiotherapy needs a paradigm shift to include biological interventions that are tailored to radiation-related phenomena
- DNA-repair mechanisms are obvious targets for interventions aimed at improving the radiotherapeutic benefit
- Chromatin structure and nuclear architecture critically influence the dynamics and extent of DNA damage and repair, and thus the response to radiation
- Cells exist along a wide spectrum of radiation responsiveness, with cancer stem cells generally being radioresistant
- Radiation therapy can be an antitumour immune adjuvant and new approaches to immunotherapy will offer the opportunity to exploit this interaction
- Biomarkers that are redox-related or immune-related might help evaluate the status of patients with cancer and provide insight into how best to combine radiotherapy with biological treatments in each individual

Review criteria

The PubMed database was searched for published, full-text articles in English. The same searches were also used to search Google Scholar, Highwire, JSTOR, and the Web of Science, and through reading articles. Main search terms were “DNA repair”, “chromatin structure”, “PARP”, “PARP inhibitors”, “non-homologous end joining”, “homologous recombination”, “senescence”, “H2AX”, “ATM”, “BRCA1”, “chromatin dynamics”, “histone deacetylation”, “HDAC Inhibitors”, “Nrf2”, “redox”, “immunity”, “macrophage subsets”, and “immune activation”. No restriction was placed on the year of publication. Broad searches were performed first before adding limiting search terms, such as “ionizing radiation”, “charged-particle therapy”, and “SBRT”.

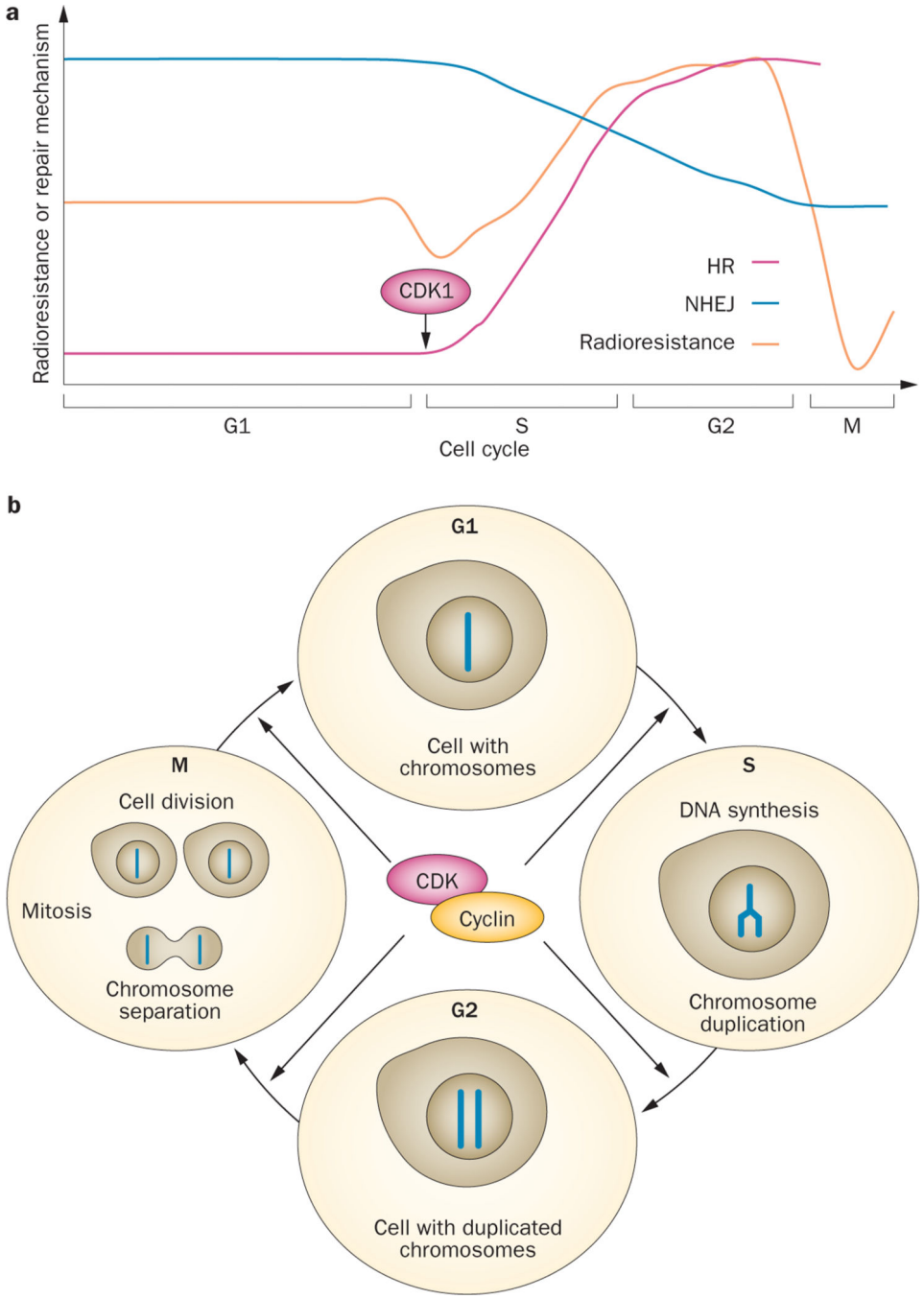

Figure 1 |.

The effect of the cell-cycle phase on radiosensitivity and on the DNA-repair pathway that is utilized. **a** | The schema shows how CDK1 engages HR in the S and G2 phase of the cell cycle, which coincides with an increase in radioresistance, at the expense of NHEJ. **b** | Progression through the cell cycle is under the control of a network of CDKs that rely on oscillating cyclin expression. Once a cell moves forward, out of G1 and into S phase, its DNA gets duplicated and therefore increasingly allowing for DNA damage repair to

follow the more accurate route of HR. Abbreviations: CDK1, cyclin-dependent kinase 1; HR, homologous recombination; NHEJ, non-homologous end joining.

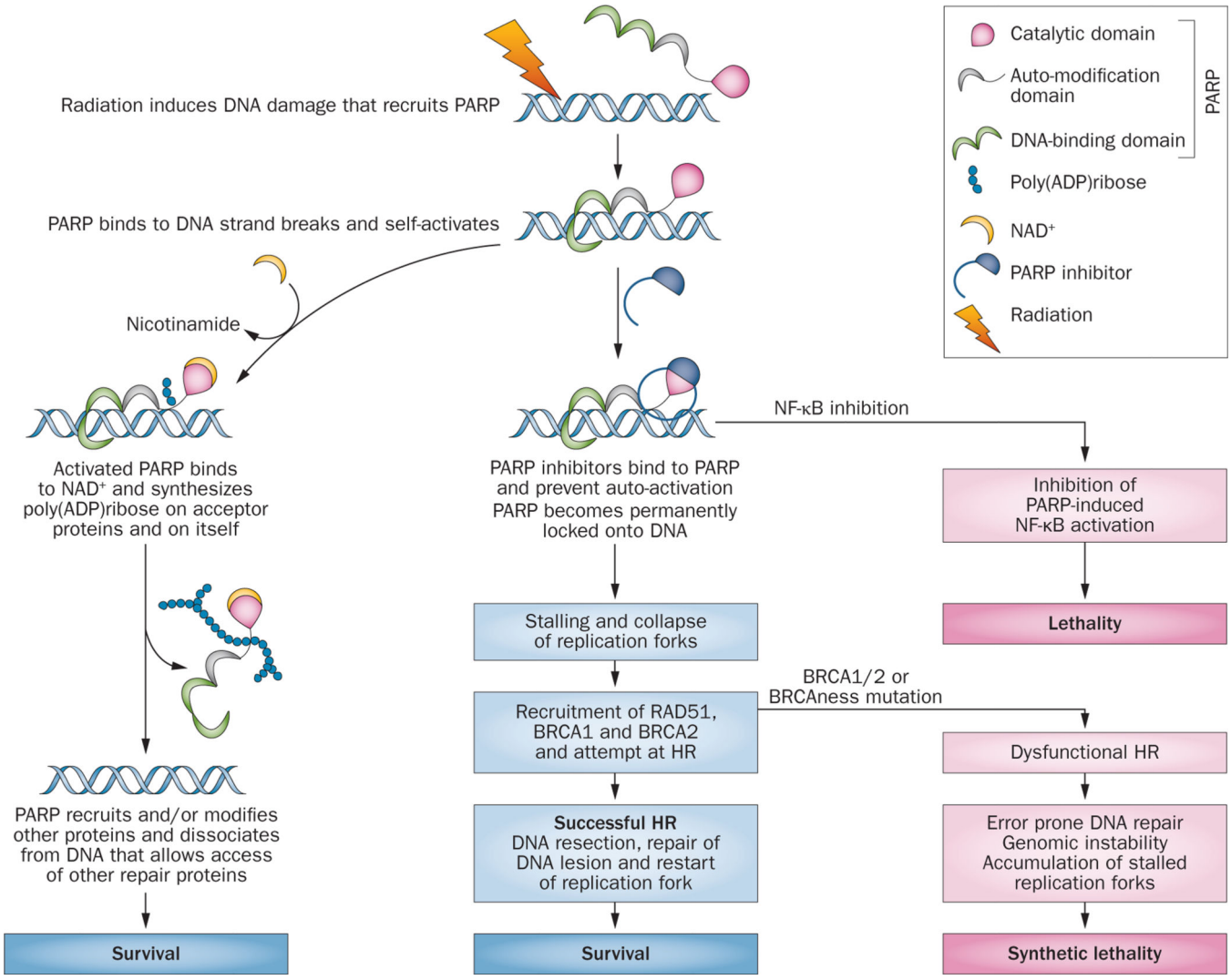

Figure 2 |.

Possible mechanisms by which PARP-1 inhibitors might interact with radiation-induced DNA damage for therapeutic benefit. PARP inhibitors cause synthetic lethality in cells that have a compromised HR apparatus or can block cell survival pathways activated through NF-κB. On the left of the diagram, once PARP disassociates from the DNA complex, recruitment of repair proteins XRCC1 and DNA ligase III for repair of SSBs by BER commences; MRE11 and ATM facilitate DSB repair through HR. MRE11 also facilitates restart of stalled replication forks. The repair proteins help regulate chromatin structure, DNA methylation, histone H1 binding to chromatin, and transcriptional regulation of survival genes, such as NF-kB. Abbreviations: BER, base-excision repair; CD, *C*-terminal catalytic domain; DSB, double-strand break; HR, homologous recombination; NF-κB, nuclear factor κB; SSBs, single-strand breaks; PARP, poly(ADPribose) polymerase.

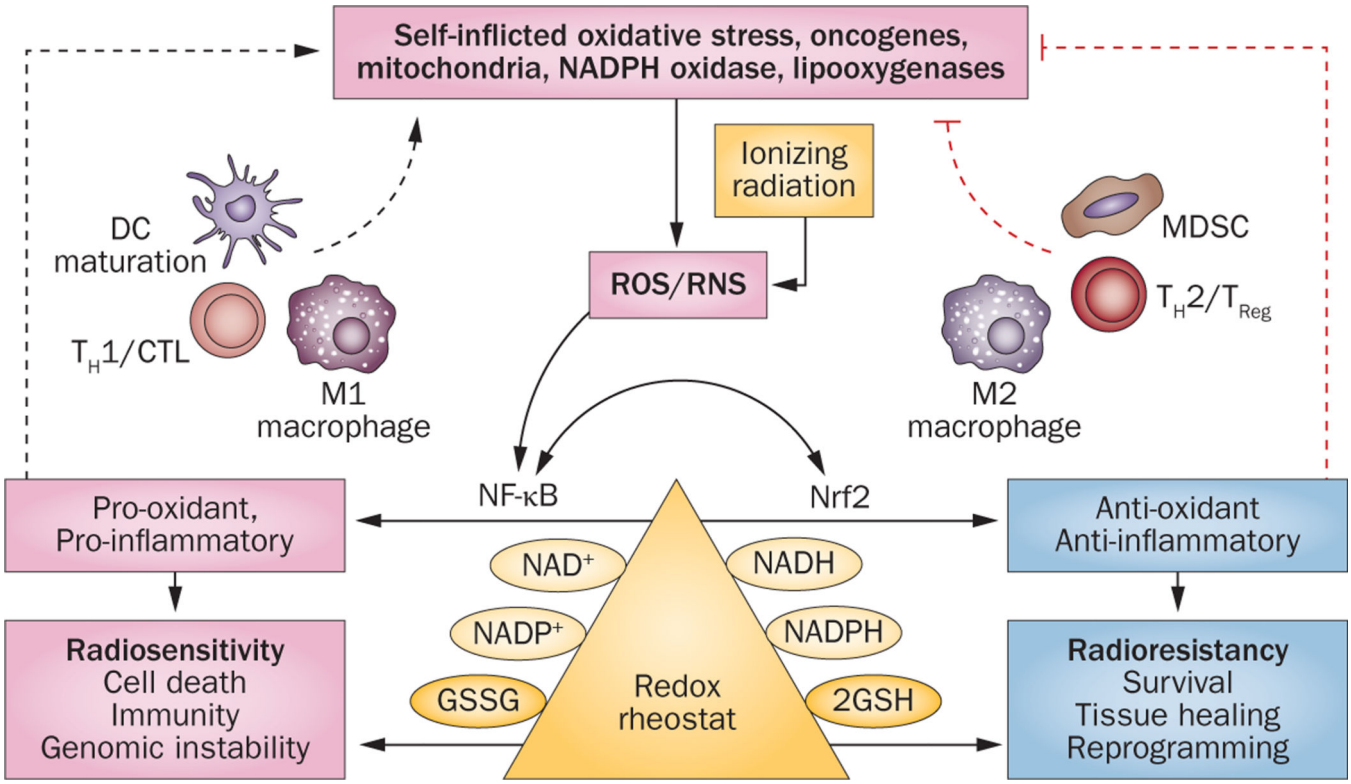

Figure 3 |.

Redox and how it might influence both radiation responses and the immune system. The concept presented is that radiation-induced ROS/RNS drives the generation of a pro-oxidant state that results in acute inflammation, proinflammatory cytokines, more ROS/RNS production, and self-inflicted oxidative damage. At the same time, ROS has the ability to promote antigen presentation by DCs, T_H1 cells, CTL and M1 macrophage responses, with further production of proinflammatory cytokines—all of which affirm the pro-oxidative state (left-hand side). The redox imbalance that is created eventually drives an antioxidant response (right-hand side) with increased glutathione synthesis, cell survival, and radioresistance. Under the same influences, immune-control mechanisms, including T_{REG} cells, MDSC, M2 macrophages, and T_H2 responses, as well as antioxidants and anti-inflammatory cytokines, will be favoured. Abbreviations: 2GSH, 2 monomeric glutathione molecules; CTL, cytotoxic T-cell lymphocyte; DC, dendritic cell; GSSG, glutathione disulphide; MDSC, myeloid-derived suppressor cells; Nrf2, nuclear factor (erythroid-derived 2)-like 2; ROS, reactive oxygen species; RNS, reactive nitrogen species; T_H1, T-helper type 1 (cell); TH2, T-helper type 2 (cell); TREG, regulatory T (cell).