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Radiation therapy-induced metastasis: radiobiology and clinical implications

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Circulating tumour cell (CTC); non-small cell lung cancer (NSCLC); 3D-conformal radiotherapy (3D-CRT); intensity-modulated radiotherapy (IMRT); volumetric modulated arc therapy (VMAT); flattening filter-free (FFF); microbeam radiotherapy (MRT); transforming growth factor- β 1 (TGF- β); matrix metallo-proteinase (MMP); epithelial-to-mesenchymal transition (EMT).

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Abstract

Radiation therapy is an effective means of achieving local control in a wide range of primary tumours, with the reduction in the size of the tumour(s) thought to mediate the observed reductions in metastatic spread in clinical trials. However, there is evidence to suggest that the complex changes induced by radiation in the tumour environment can also present metastatic risks that may counteract the long-term efficacy of the treatment. More than 25 years ago, several largely theoretical mechanisms by which radiation exposure might increase metastatic risk were postulated. These include the direct release of tumour cells into the circulation, systemic effects of tumour and normal tissue irradiation and radiation-induced changes in tumour cell phenotype. Here, we review the data that has since emerged to either support or refute these putative mechanisms focusing on how the unique radiobiology underlying modern radiotherapy modalities might alter these risks.

MANUSCRIPT TEXT

The idea that radiation therapy used to treat tumours might also contribute to tumour metastasis is not a new one, having been described in pre-clinical and clinical studies for at least 60 years [1], alongside effects documented for other cancer treatments and interventions, reviewed in [2]. In a comprehensive review of radiation-induced enhancement of metastasis in 1991, von Essen described four mechanisms that might be considered to influence radiation-induced metastasis [3], namely: radiation-induced release of tumour cells into the circulation; effects at distant normal tissue sites which might host metastases; radiation-induced changes in surviving tumour cells; and, local control-mediated prolonging of metastatic cell release. Although our understanding in these areas has grown considerably, these same four prospective modes of radiotherapy-mediated effects on tumour metastasis remain relevant in current discussions on the topic. While the evidence available more than 25 years ago to support the mechanism of radiation-induced circulating tumour cell (CTC) release was limited to *in vitro* proof-of-principle and experiments in animal models of transplanted tumours, our group recently reported that early in the course of radiotherapy, some patients with non-small cell lung cancer (NSCLC) showed release of CTCs or clusters containing multiple CTCs into the circulation [4]. Other clinical evidence supporting the effect of radiotherapy on distant metastasis has also begun to mount, reviewed in [5]. In the time since von Essen outlined the above-mentioned four modes of radiation-induced metastasis, not only has there been much work into investigating these mechanisms, utilising advances in cellular and molecular biology, but the nature of radiation therapy itself has also changed dramatically. Here, we will discuss the progress that has been made in the investigation of these mechanisms, with particular focus on the changing radiotherapy landscape and the impact of modern and future radiotherapy modalities.

The Changing Landscape of Radiation Therapy

Radiation therapy remains the most effective non-surgical technique to achieve control of malignant tumours [6]. It can be utilised at each stage of the patient journey to: downstage primary tumours, reduce the risk of recurrence in the adjuvant setting, and in palliative settings to improve symptoms and quality of life. Whilst radiation therapy has been in use for over a century, the past two decades have seen an exponential increase in technological advances aimed at improving tolerability, accuracy and efficacy.

Technical advances have been made in both treatment planning and delivery. Increased sophistication in imaging and treatment planning system algorithms allow for ever-increasing ease and precision in localisation and

demarcation of the target volume, providing the input for the integrated radiation delivery systems. However, developments in precision ultimately reach a plateau as the dose and physical uncertainties fall below the biological uncertainty of where the optimal lines should be drawn, such that the largest source of error becomes doubt in how to optimally define the target volume to achieve maximal biological response. The improvements in treatment planning and associated physical-spatial-temporal models allowed the evaluation of all possible permutations for how to deliver the desired dose to the target volume, leading to novel technological and strategic approaches to the physical delivery of photon irradiation [7,8], such as 3D-conformal radiotherapy (3D-CRT), intensity-modulated radiotherapy (IMRT) and volumetric modulated arc therapy (VMAT). However, where the desired dose-distribution to the target entered into the models is the same, these varied approaches all ultimately achieve similar irradiation of the target volume, but with various compromises in the dose distribution in the surrounding normal tissues [9,10]. The relative advantages of these competing photon radiotherapy technologies thus depend on whether minimizing total dose, maximum dose or irradiated volume in normal tissues is preferable (a question of ongoing debate, [11]), and other factors such as cost, ease and flexibility. With increased ability to spare normal tissues, these novel photon radiotherapy modalities can also allow for increasing tumour doses and altering other treatment parameters [12,13], in line with results from clinical trials [14].

Novel radiotherapy modalities such as stereotactic radiotherapy, brachytherapy, and proton and carbon ions are also becoming increasingly prevalent, reviewed in [15]. Historically, radiotherapy has been delivered in a fractionated manner with five daily fractions per week each of 2 Gy, with the convenience of such schedules overlapping with the justification of allowing time between fractions for normal tissue recovery. Coupled with the benefits to sparing of normal tissue, fractionation can also have benefits in terms of tumour control including: limiting repopulation, improvement in oxygenation and allowing for cell cycle changes whereby tumour cells can move into the more sensitive G2/M phase of the cell cycle, reviewed in [16]. Such fractionation has been used since Coutard proposed it in 1934 [17], with the linear quadratic cell-killing model and derived alpha/beta (α/β) ratios underpinning the radiobiological effects of fractionation on tumours and normal tissues [18]. Yet despite their sparing effects of normal tissues, increased fraction delivery time and duration of treatment course have been implicated in reduced treatment efficacy [19,20], allowing for repopulation and recovery of tumour cells via sub-lethal damage repair. Accelerated radiotherapy has been found to show improvements in local control in head and neck cancer [21] and increases in overall survival in lung cancer [22,23]. Conversely, the delivery of larger fraction doses in stereotactic treatments often requires intra-fraction imaging with cone-beam computed tomography (CT) or other imaging techniques, which may prolong fraction delivery. By decreasing fraction

delivery times [24], the use of VMAT and flattening filter-free (FFF) treatment aim to negate potentially detrimental biological consequences including intra-fraction motion and tumour recovery. Clinical trials of stereotactic radiotherapy utilise a variety of fractionation regimens based on tumour volume, site of disease and proximity to organs at risk [25-27]. Despite the higher radiation levels delivered to the tumour, there may only be limited increases in cell kill due to the higher α/β ratio of hypoxic clonogens [28], while losing the benefits of re-oxygenation observed with conventional fractionation. Many studies have reviewed the applicability of the linear-quadratic model to hypo-fractionation with no clear consensus on its validity [18,29,30].

Beyond fractionation, there are other exposure parameters related to modality and delivery that may impact on the cells' individual responses to treatment, including radiation quality and dose-rate (Figure 1). Charged particle therapies, primarily proton and carbon ion beams, take advantage of the favourable physical properties of charged particles which deposit dose in a sharp and narrow peak close to the end of their range, reviewed in [31,32]. The lower entrance doses and minimal exit doses mean that the dose delivered to organs at risk is significantly lower than for external-beam radiotherapy techniques. The relative biological effectiveness of protons is marginally higher than reference X-rays (particularly at the end of the track, [33]) but markedly increases with the use of heavier ions, preferentially within the target volume. These differences in both dose localisation and relative biological effectiveness, as well as interactions with tumour hypoxia [32], mean that the radiobiology of charged-particle therapies could have unforeseen implications for radiation-enhanced metastasis [34].

Brachytherapy uses implanted radioactive 'seeds' to deliver higher biologically-effective doses close to the tumour tapering off to low doses at the borders with surrounding tissues, and has been shown to be effective in curing low-risk prostate cancers and is a key component of the management of gynaecological malignancies. One of the attractive features of brachytherapy is the ability to deliver a high dose-rate [35], a feature also shared with FFF treatments (with average dose rates of up to 0.5 Gy per second, [36]) and proton pencil beam scanning which can reach up to Gy per millisecond dose-rates [37]. Recently, small animal irradiation platforms delivering up to 50 Gy per second photon irradiation (so-called FLASH irradiation protocols) have shown optimal tumour control and a reduction in normal tissue toxicity [38], furthering interest in the development of clinical linear accelerators able to achieve such dose-rates. A new modality known as microbeam radiotherapy (MRT), in which the high dose-rate X-ray beam is split into an array of planar parallel microbeams, has shown promise in sparing normal tissues while still achieving tumour cell kill [39,40]; although, it is currently only achievable in synchrotron facilities. Although clinical implementation of MRT is some time away [41], it is considered to be a technically

feasible alternative to conventional broad-beam radiotherapy.

Whilst these various novel treatments offer potential improvements in side-effect profiles and improved efficacy for tumour control, the radiobiological implications of the altered radiation exposure parameters for the tumour and its microenvironment remain under investigation. Although a key element of the consent process for any cancer intervention, including radiotherapy, is informing patients of the risks and side effects of the treatment, it is an implicit assumption that the proposed treatment will not increase the chances of metastatic disease, often the ultimate cause of cancer mortality. The unfavourable propositions that an intervention to achieve cure can simultaneously disseminate malignant cells into the circulation, and/or increase the metastatic potential of any remaining tumour cells, are however plausible ones.

Release of Tumours Cells into the Circulation

Many patients succumb to distant metastases despite optimal loco-regional control of primary lesions (Figure 2), primarily attributed to so-called occult micro-metastases which were already present at the time of diagnosis [42-44]), with subsequent growth at these distant sites appearing once a supportive vascular network is established. An alternative, and non-exclusive mechanism is that malignant cells may also be released into the circulation by iatrogenic (caused by a medical intervention) disruption of the tumour. Ebos drew a parallel to Hercules' battle with the Lernaean Hydra in Greek Mythology i.e. for each head cut off, two more sprung up to replace it [2]. It is possible that pre-existing or treatment-induced metastasis may be responsible in different patients, or even occur in parallel in the same patient. It is also plausible that radiation treatment of pre-existing metastatic lesions may spawn the spread of further metastases.

All treatments for cancer including surgery, chemotherapy and radiotherapy have been implicated in changes to the cancer cell and its environment which can release tumour cells into the surrounding vasculature and enable tumour cells to develop phenotypes which can lead to metastatic spread, reviewed in [2]. A number of pre-clinical studies have clearly indicated the potential for radiation to enhance metastatic disease [45], with a variety of mechanisms now clearly established (Figure 3). Despite this, the clinical data from human studies has failed to uncover a clear pro-metastatic effect, most likely due to the diversity of human tumour responses. This was illustrated in a mouse xenograft model, where rhabdomyosarcoma lines derived from two different patients showed opposing effects of local fractionated irradiation to the subcutaneous implants on metastatic spread [46].

Reviews of a range of studies into the prognostic significance of CTC levels have concluded that the number of CTCs is an independent predictor of distant metastasis [47], but whether the most relevant time-point for analysis is before, during or after treatment is still unclear. A study comparing CTC release in uveal melanoma patients undergoing different treatments showed that 3 out of 49 patients undergoing stereotactic radiotherapy were positive for CTCs post-treatment, compared to 0 out of 15 patients treated with brachytherapy [48], but more data in this area are needed before direct comparisons can be made, particularly given the immense challenge of detecting rare CTCs [49]. A study in NSCLC patients treated with proton therapy or intensity-modulated radiation therapy demonstrated a trend towards decreasing CTC counts during and following either radiotherapy modality [50]. In one case, high CTC levels during treatment increased further following radiotherapy, with the patient soon diagnosed with distant failure. In a mouse model, irradiation of sub-cutaneous implanted melanomas with high dose-rate gamma-rays (2.5 Gy/min) was more effective at both local control and reduction in metastasis to the lungs compared with the same dose delivered at low dose-rate (40 mGy/min), although both dose-rates reduced metastasis compared to unirradiated animals [51]. However, whether such differences are relevant to the much higher dose-rates now coming into use in clinical radiotherapy is unclear, as is whether the reduced metastatic risk is a secondary measure of improved local control or includes an effect of treatment on metastasis directly. The development of mouse models for testing relevant radiotherapy treatment plans [45], including stereotactic radiotherapy in oligo-metastatic disease [52] will assist in answering such questions. The risk associated with potential CTC release induced by ablative hypo-fractionated doses delivered to metastatic sites is unclear, especially where such treatments are non-curative [53]. There are currently no data available on the ability of modalities such as carbon ion therapy, FLASH radiotherapy or MRT to induce the release of tumour cells into the circulation. Despite the abundance of clinical trials into various radiotherapy modalities, regimens and combined-therapies, there is still a significant lack of knowledge as to how the parameters of dose, dose-rate, fractionation and radiation quality influence any radiotherapy-enhanced CTC release. Since local control and side-effect profiles are the primary metrics for comparing novel radiotherapy treatments against current standard-of-care, and distant-metastasis (either spontaneous or treatment-induced) is already monitored, it is not expected that the potential for CTC release alone would influence the selection of any one modality in the absence of inferior metastatic outcomes. Yet, if radiotherapy-induced CTCs were found to be significantly higher for an otherwise attractive modality, it represents a potential target for further improving the long-term success of such treatments.

Effects in Irradiated Non-Tumour Cells and at Distant Non-Irradiated Sites

Another of the mechanisms for radiation-enhanced metastasis hypothesised by von Essen, was that radiation might influence metastatic risk indirectly through effects on non-tumour cells. Evidence for radiation-induced bystander effects suggests that cells in an irradiated volume can induce biological changes in neighbouring unirradiated cells by inter-cellular communication, reviewed in [54,55], an effect that appears to be highly dependent on radiation quality with implications for charged particle therapies [56]. Lethally-irradiated tumour cells have long been shown to be able to promote malignant growth when mixed and co-injected with non-irradiated viable cells in animal models [57,58], an effect not observed with heat-killed tumour cells. It has also been demonstrated that the normal tissue/stroma surrounding an irradiated tumour may become both more hostile to a re-growing tumour and more permissive to tumour cell escape, known as the tumour bed effect, reviewed in [59-61]. This is particularly relevant when radiotherapy is used in an adjuvant setting, such as after breast conservation surgery. In this case, irradiation of the presumptive normal breast surrounding the tumour excision site is effective at preventing local recurrence [62], presumably mediated by eradication of invasive tumour cells that remain outside of the surgical margins. However, radiation-induced pro-metastatic effects on the normal breast tissue could still be an issue, potentially reducing the efficacy of such treatments particularly given the high rates of ipsilateral recurrence [63].

The systemic extrapolation of bystander effects, known as the abscopal effect, involves physiological changes induced by irradiation of a defined volume [64], and is usually associated with anecdotal descriptions of responses in tumours distant from the primary irradiated site, reviewed in [65,66]. Yet, mechanisms of a pro-metastatic nature have also been described. Radiation treatment to a subcutaneously-implanted tumour in mice led to the activation of dormant micro-metastases [67] via the removal of the suppressive effects of angiostatin which had been secreted by the tumour, consistent with what had been observed in anecdotal cases following surgical removal of a primary tumour. It has since been shown that growing tumours can induce a wide range of systemic effects via plasma cytokines resulting in inflammation and DNA damage at distant sites [68], responses which might be further altered by radiotherapy-induced tumour cell kill. Even irradiation of normal tissue increases the plasma concentration of circulating transforming growth factor- β 1 (TGF- β), corresponding to increased metastatic spread in a transgenic breast cancer mouse model [69], while in another model plasma interleukin- 1β (IL- 1β) levels rose during radiotherapy treatment [45], a cytokine which when delivered at high dose was extremely pro-metastatic. Similar conflicts as those observed between pro- and anti-metastatic effects of local irradiation have also been described in chemotherapy models of treatment-induced metastasis [70,71].

Abscopal effects mediated by the immune system have been explored for potential clinical exploitation [72,73]. Such effects are implicated in results showing that carbon ion irradiation of a primary implanted tumour in mice prevents the growth of a second tumour after inoculation challenge [74], with the suggestion that heavy ions might have an inherent advantage for such mechanisms [75]. Evidence for immunogenic activation of anti-tumour defences has also been observed for proton irradiation [76]. It is thought that tumour vascular damage due to the high doses used in stereotactic radiotherapy may contribute to its effectiveness in local control [77], and might similarly aid the release of immunogenic antigens triggering enhanced systemic immune responses [78]. But, mechanisms that release immunogenic antigens into the circulation might simultaneously permit the increased release of viable tumour cells into the circulation, demonstrating the conflict between pro- and anti-metastatic effects of radiotherapy. Further, hypoxia induced by sub-lethal tumour irradiation in a xenograft model actually increased metastasis compared to unirradiated tumours [79], while radiotherapy in patients with hepatocellular carcinoma seemed to enhance tumour progression outside the treatment field via induction of angiogenesis [80], adding further complexity to the extrapolation from biological observations to their final impact on metastasis risk.

The increasing range of photon delivery modalities with their accompanying variation in normal tissue irradiation may have implications for signalling between the tumour and surrounding stroma, vasculature and lymphatics. Radiation damage in non-target tissues, such as lung inflammation and fibrosis following breast tumour radiotherapy, may alter the risk of metastasis to these tissues [81]. However, since collateral damage to normal tissues scales with distance from the target volume, such sites might be at greater risk simply due to proximity to the primary tumour, complicating retrospective analysis of the locations of metastatic lesions. There is evidence that bone marrow-derived cells form a pre-metastatic niche prior to the arrival of tumour cells that can recruit and retain circulating tumour cells [82] in sites corresponding to the commonly observed metastatic profile of a given tumour. It is possible that radiation exposure of either the bone marrow or the pre-metastatic niches could alter this process, however it is unclear whether such effects would be pro- or anti-metastatic.

Co-culture of pancreatic cancer cells with gamma-irradiated stromal fibroblasts increases their invasiveness through secretion of paracrine signals [83], with alterations in the extracellular matrix responsible for increased invasiveness of breast tumour cells after irradiation of the basement membrane [84]. Irradiated tumours themselves may secrete signals that recruit tumour cells already in the circulation back to the primary tumour site [85]. Many more such radiation effects on the tissue microenvironment have been described, reviewed in [86],

and may contribute (positively or negatively) to the incidence of metastasis from irradiated tumour sites, and each may be sensitive to the changes in irradiation parameters that accompany the various radiotherapy modalities. For example, experimental evidence that carbon ion [87] and proton irradiation [88] suppresses angiogenic factors in non-transformed cells suggests a mechanism by which radiation quality could impact the receptiveness of surrounding tissues to pre-existing or incoming tumour cells.

Radiation-Induced Changes in Surviving Tumour Cells

At the time of von Essen's review, there was limited evidence supporting the proposed hypothesis 'direct alteration of tumour cells by irradiation.' Although radiation exposure can cause a wide range of changes within cells that can lead to a variety of responses and cell fates, reviewed in [89], radiobiology research over the past century has been dominated by two primary endpoints: cell death and mutation. These principal measures of radiation effect are driven by context, with radiation-induced cell death the most relevant metric for both treatment success and normal tissue complications in radiotherapy, while DNA mutations are assessed as the mediators of radiation-induced cancer risk. This convergence to ultimately express fractions of live versus dead cells, or mutant versus normal cells as the prime measures of radiation effect has meant that radiation-induced metabolic changes, gene-expression responses, or phenotypic changes such as motility, activation, proliferation and signalling are less-well understood, and were rarely regarded as primary outcomes of radiation exposure.

Yet as the context changes, so do the radiation-induced effects which become most relevant. With the increasing success of radiotherapy in controlling primary tumours, the effects radiation has on surviving tumour cells come to the fore when considering post-treatment secondary cancer/metastatic risk [90]. One caveat is the effect of selection, where radiation may not induce a phenotypic change, but it might indirectly result in a surviving population with a distinct phenotype from that which was predominant in the initial population. Indeed, radiation-induced selection may be the major mechanism underlying changes in tumour cell phenotype caused by radiotherapy [91]. This is key to the question as to whether radiation-induced cell death of sensitive subpopulations passively results in the selection of radioresistant tumour cells, or whether radiation actively promotes features of radioresistance [92]. An example of the latter is radiotherapy-induced hypoxia [93] that alters the inherent radioresistance of the remaining tumour cell population, or the radiation-induced induction of cathepsin S, a cysteine protease, in breast cancer cells which increases their radioresistance [94].

Where radiation eliminates or permanently incapacitates cells, either through direct or delayed cell death or senescence/differentiation, short-term observations in irradiated cell populations may not correspond to the

changes observed in long-term survivors [95]. However, transient phenotypic changes may nonetheless influence metastatic risk where tumour cells are released during radiotherapy while still expressing a transient radiation-induced phenotype; where short-term responses aid inter-fraction recovery or proliferation; or, where radiation-induced changes in tumour cells permanently alter their surrounding microenvironment. This last case blurs the lines between radiation effects in tumour and non-tumour cells, with radiation-induced angiogenic signalling from tumour cells able to stimulate the local vasculature at the same time as radiation exposure has its own direct effects on the vasculature [77,60]. Interestingly, such complex interactions may differ with radiotherapy modalities, with carbon ion and proton irradiation both shown to suppress angiogenesis in irradiated tumour cells, unlike pro-angiogenic responses observed for photon irradiation [96,88].

It is also important to differentiate between radiation-induced phenotypic changes which are generalizable and repeatable, and changes due to stochastic radiation-induced mutations in surviving tumour cells. This also applies more generally to the variability of human cancers where, as shown in a study by Fujita et al. [97], panels of cell lines fell on a spectrum from radiation-induced to radiation-reduced invasiveness, with the genetic status of given tumours shown to alter their response to radiation [98]. There may not be a single answer as to whether a radiotherapy modality ultimately promotes or inhibits the metastatic properties of a tumour, with the result dependent on the nature of the patient's tumour.

Thus, it is necessary to consider what changes radiation might induce in tumour cells which will ultimately survive a course of radiotherapy treatment, or survive until being released as CTCs. The specifics of such changes are themselves likely to be dependent on the radiation exposure scenario, with repeated exposure to fractionated radiotherapy across several cellular generations potentially inducing different responses to a single high radiation dose, while increasing dose-per-fraction or decreasing inter-fraction time might decrease the viability of any tumour cells released as CTCs during the initial fractions of radiotherapy treatment [22]. The use of concomitant chemotherapy to radiosensitise tumour cells could reduce the risk of viable CTCs entering the bloodstream early during the course of radiotherapy, with some evidence of reduced distant-metastasis with concomitant cisplatin compared to radiotherapy alone [99], as well as a combination of cisplatin and a hypoxic radiosensitiser [100]. A meta-analysis of cervical cancer trials using both platinum and non-platinum based chemoradiotherapy [101] showed evidence of reduced metastatic risk and delayed metastatic interval, consistent with such a mechanism.

The discovery that sub-lethal gamma-irradiation of cultured malignant glioma cells increased features of motility, adhesion and cell-cell interactions to produce a more invasive phenotype, which was confirmed by increased

invasiveness when implanted into mice [102], provided some of the first evidence for the ability of radiation to alter the metastatic properties of tumour cells. Shortly thereafter, similar findings in pancreatic tumour cell lines exposed to 3 – 10 Gy of gamma radiation showed increased invasiveness, similarly with evidence for up-regulation of matrix metallo-proteinase (MMP) activity [103]. Yet already, these studies began to show that responses were not uniform, with responding and non-responding lines, and different outcomes between migration, motility and invasiveness which did not always correlate. The plethora of evidence which has since been accumulated in this area has been reviewed in detail [104,97]. Importantly, studies have since confirmed that these phenotypic changes (motility, invasiveness and MMP expression) exist in the long-term survivors after high-dose irradiation [95].

Of great interest were findings that irradiation with protons or carbon ions did not induce the invasive phenotype observed with sub-lethal doses of photon irradiation [105,106], and unlike photons, inhibited MMP expression. Later work showed that although increased proliferation and invasiveness could be observed in some cell lines after X-ray irradiation, both were decreased in the same cells when exposed to carbon ion irradiation [107]. Interestingly, increasing the X-ray dose further showed similar decreases in proliferation and invasiveness to that observed with the heavy ions, suggesting hypo-fractionated irradiation may induce different responses. This is supported by recent work demonstrating a range of differences in the biological responses of lung cancer cells to stereotactic versus conventionally-fractionated radiotherapy doses [108]. Further work in medulloblastoma cell lines showed that both photon and carbon ion irradiation decreased motility and down-regulated MMP expression [109], showing again that pro-metastatic changes are not universal or strictly predicted by radiation quality, reviewed in [110]. Additional mechanisms underlying these changes have been discovered including changes in the actin cytoskeleton and stiffness [111,112]; increased integrin expression [113]; increased oncogene transcription [114]; and, increased secretion of lysyl oxidase [115]. Improved understanding of the mechanisms underlying these effects will help to determine which effects are most relevant to human radiotherapy scenarios.

The findings which have attracted the most attention have been those related to the ability of radiation to induce or promote epithelial-to-mesenchymal transition (EMT) in tumour cells, which was the subject of a recent comprehensive review by Lee et al. [116]. It has been unclear whether the EMT-like changes observed are due to the precocious induction of the spontaneous EMT observed in a variety of human cancers, or whether specific biological responses which overlap with spontaneous EMT are independently invoked. Whether the EMT/EMT-

like changes are independent of the motility, migration and invasion phenotypes mentioned above, or whether they represent various aspects of a broader program is also yet to be resolved.

Key steps forward in this area have come from experiments showing that irradiated tumour cells show not only changes in EMT-markers, but functional changes that demonstrate the acquisition of cancer stem-cell properties [117,118]. Cancer stem-cells are known to be inherently radioresistant, due in part to an enhanced DNA damage response [119,120]. Radiation has been shown to induce gene expression changes similar to patterns observed in embryonic stem cells [121], and irradiated breast tumour cells show complex temporal changes in the expression of EMT regulators such as Bmi-1, vimentin and E-cadherin [122], with initial decreases in invasiveness followed by gradual elevations to above-baseline levels. Changes in chromatin configuration have emerged as essential to EMT-related transcription factor regulation [123,124], but they are still to be fully characterised. Weyemi et al. discovered that down-regulation of H2AX, a histone variant involved in DNA repair [125], can regulate EMT and its reversal by changing chromatin configuration and by controlling the critical EMT-associated transcription factors Slug and ZEB1 in colon carcinoma cells [126]. A further report in human mammary MCF10A cells [127] validated the important role of H2AX levels in the tissue-specific regulation of EMT (Twist1/Slug for breast; ZEB1/Slug for colon), with the *in vitro* and *in vivo* evidence reviewed in [128]. Chromatin changes in the wake of tumour cell irradiation present an attractive link between radiotherapy and the induction of EMT, but further experiments are needed to characterise these connections.

Models for tracking metastasis and EMT changes *in vivo* following radiotherapy provide a method to further explore the nature of the phenomenon [129], including experimental systems that allow *in vivo* testing of stereotactic radiotherapy regimens [52]. Two different studies showing the induction of EMT after fractionated radiotherapy [130,131] have demonstrated the relevance of the effect to routine human cancer therapy, with important implications for disease monitoring where radiation exposure can depress tumour markers being used as clinical biomarkers. Further experiments have implicated yet more mechanistic links, including TGF- β expression [132] and signalling through the bioactive gas hydrogen sulphide [133], reviewed in [134]. It should be noted that radiation-induction of EMT is also not universally observed [135], with evidence that the cell culture systems can interfere with altered morphology associated with EMT, highlighting the need for caution in generalising these phenomena from the specific observations to recommendations for cancer therapy strategies. The range of radiation-induced biological effects in tumour cells continues to grow with observations of altered antigen expression in tumour cells [136] and changes in differentiation [137]. Ever-expanding ‘omics’ techniques

will no doubt increase the number of biological effects found to be induced (or suppressed) in irradiated tumour cells into the future.

Potential for Intervention

Given our current limited understanding of the precise mechanisms which might facilitate radiation-induced metastasis, it is difficult to nominate general interventions which would be of certain benefit in reducing the risk of distant metastasis following radiotherapy. For instance, although the immune system has been shown to play a critical role in eliminating disseminated tumour cells [138], it is unclear whether promoting a generic pro-inflammatory environment would be beneficial overall given counteracting effects on normal tissues which might promote a receptive environment for a metastatic niche [81]. Yet, better understanding of specific immune pathways allows for targeted approaches, such as following radiotherapy (known to up-regulate the expression of the immune suppressive PD-L1 in tumour cells [139]) with a PD-L1 inhibitor, which prevented and delayed metastasis [140]. This paradigm begins to blur the line between immunotherapy as a secondary compliment to curative radiotherapy, and radiotherapy as an adjuvant prior to curative immunotherapy [141]. Such an approach is the use of pre-surgical neoadjuvant radiotherapy to stimulate anti-tumour immune responses [142]. Alternative proposals include the use of accelerated radiotherapy to reduce the viability of CTCs released at the start of radiotherapy [5], or the use of thrombolytic agents to destabilise CTC clusters [143]. The design of such specific interventions relies on the capture of quality data during clinical trials, such that local failure can be clearly differentiated from distant metastasis, and the timing and location of metastasis can be interrogated in more detail. These data can form the basis of sophisticated models that allow both the testing of specific hypotheses [144,145], and the discovery of new relationships between radiotherapy treatment and metastasis that can provide new targets for intervention.

Conclusion

Radiation therapy clearly reduces the risk of metastasis compared to administering no treatment. The question at stake is whether the efficacy of the treatment, which inhibits metastasis primary through the killing of tumour cells in the treatment volume, is counteracted (to a variable extent) by radiation-induced effects which might disseminate viable tumour cells, have systemic pro-metastatic effects through signalling, and/or alter the phenotype of the cells which survive the radiation treatment. Any measurement of metastasis rates in a radiotherapy clinical trial will represent the net of the desired anti-metastatic effect and any undesirable pro-

metastatic effects, so it is not a simple task to separate these potentially conflicting processes. Improvements in long-term treatment success could be made both through further enhancing tumour cell kill and local control, but also, through preventing any radiotherapy-induced metastatic mechanisms, which may be specific to the different treatment modalities. Comparisons of distant metastasis-free survival rates between radiotherapy treatment modalities may not immediately reveal any pro- or anti-metastatic events that vary with irradiation parameters such as dose, dose-rate or radiation quality, but basic radiobiology research and improving animal models are beginning to uncover the complexity across the tumour microenvironment. Further understanding the variables that contribute to these treatment-induced metastasis risks is key to designing appropriate prevention and mitigation strategies, and identifying modalities which may have inherent advantages for preventing distant relapse.

FIGURE CAPTIONS

Figure 1 Key Radiation Exposure Parameters Defining Modern Radiation Therapy Modalities

Compared to conventional radiotherapy (photons, 5×2 Gy fractions per week) many modern radiation therapy modalities change one or more exposure parameters with the goals of: improving local control, sparing normal tissue, improving patient experience (shorter overall treatment times) and/or reducing the risk of radiation-induced second malignancies. The term ‘novel photon modalities’ includes a variety of methods including, but not limited to: 3D-conformal radiotherapy, intensity-modulated radiotherapy, volumetric modulated arc therapy and flattening filter-free radiotherapy.

Figure 2 Mechanisms of Post-Treatment Distant Metastasis

In some clinical cases, although imaging after treatment of a localised primary tumour (A, arrow) shows complete local control, disseminated metastases can be observed (B, star) several months after the completion of treatment, which were not evident in prior imaging (in mediastinum, lung, liver and vertebrae). There are several explanations for such phenomena (C): the first is that spontaneous metastasis from the primary tumour occurred prior to treatment, but were below the level of detection, so-called occult micro-metastases; another explanation is that spontaneous metastasis from the primary tumour occurred in spite of radiation treatment; however, the possibility is now being actively explored that radiation treatment might play a role in cases of rapid dissemination, even while simultaneously achieving complete local control.

Figure 3 Radiation Effects on the Tumour Microenvironment

Radiation exposure has a variety of effects on tumours and their local environment. (1) Radiation causes extensive tumour cell death. (2) Irradiated tumours and surrounding stroma may suffer physical disruption. (3) Radiation can damage the tumour vasculature. (4) The disturbance of the tumour and vasculature can result in the release of tumour cells into the circulation. (5) Radiation can have direct effects on surviving tumour cells, altering their phenotype, such as altered migration, invasiveness, radioresistance, hypoxia, epithelial-to-mesenchymal transition, and stemness. (6) Radiation selects for pre-existing radioresistant cells and cancer stem cells which now represent more of the total tumour cell population. (7) Radiation can alter the phenotype of irradiated stroma. (8) Radiation can induce signalling from the surrounding stroma into the local area and into the circulation. (9) Irradiated tumour cells can also release signals into the local area and into the circulation. Signalling molecules implicated in radiation-induced metastasis include but are not limited to VEGF, LOX, HIF-1, EGFR and TGF- β .

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