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## SMALL ANIMAL IGRT SPECIAL FEATURE: REVIEW ARTICLE

# The changing paradigm of tumour response to irradiation

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### ABSTRACT

Tumours contain multiple different cell populations, including cells derived from the bone marrow as well as cancer-associated fibroblasts and various stromal populations including the vasculature. The microenvironment of the tumour cells plays a significant role in the response of the tumour to radiation treatment. Low levels of oxygen (hypoxia) caused by the poorly organized vasculature in tumours have long been known to affect radiation response; however, other aspects of the microenvironment may also play important roles. This article reviews some of the old literature concerning tumour response to irradiation and relates this to current concepts about the role of the tumour microenvironment in tumour response to radiation treatment. Included in the discussion are the role of cancer stem cells, radiation damage to the vasculature and the potential for radiation to enhance immune activity against tumour cells. Radiation treatment can cause a significant influx of bone marrow-derived cell populations into both normal tissues and tumours. Potential roles of such cells may include enhancing vascular recovery as well as modulating immune reactivity.

### INTRODUCTION

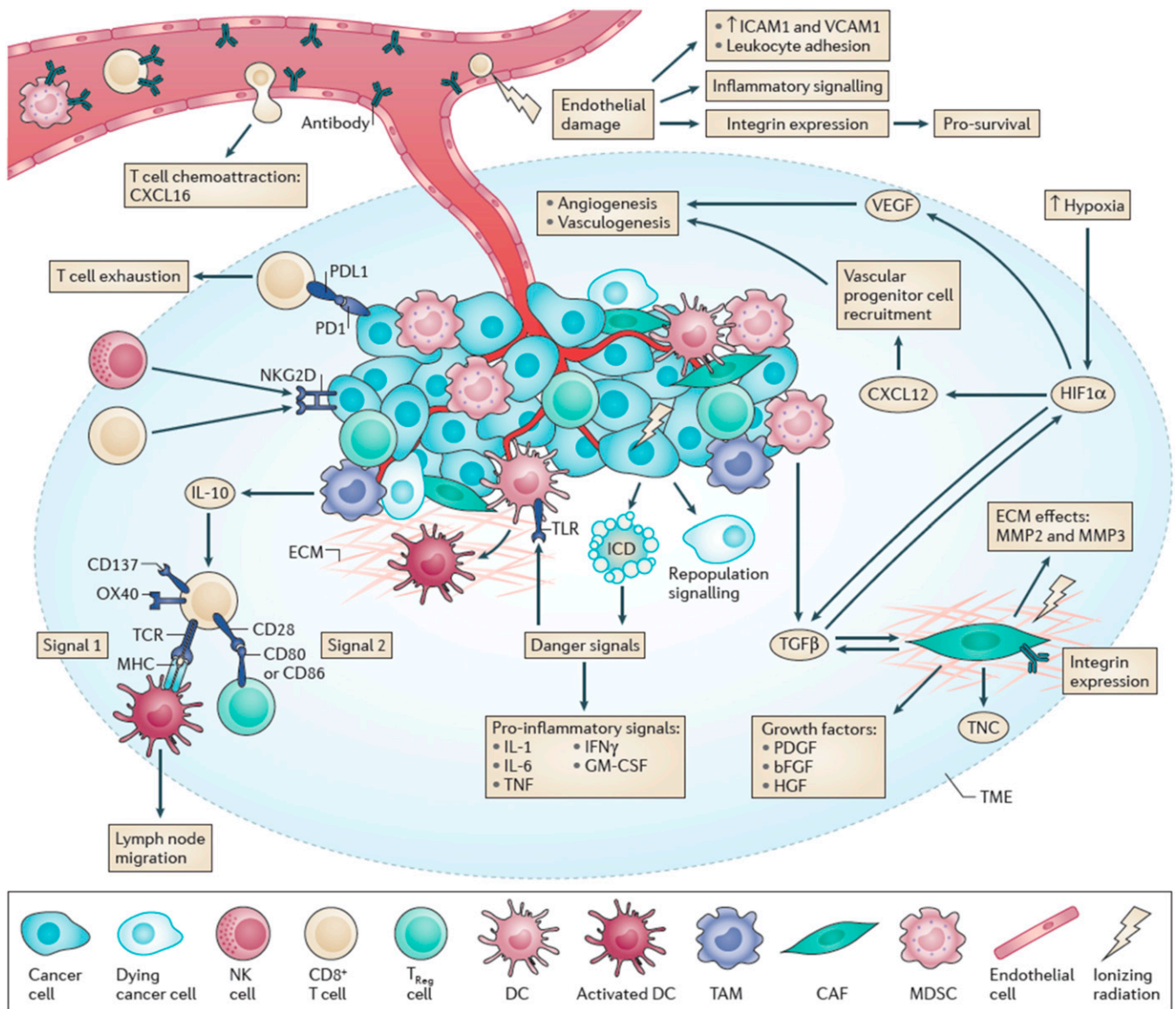
The response of tumours to radiation treatment is multifactorial and depends on features of the tumour microenvironment as well as the intrinsic sensitivity of the tumour cells themselves. Tumours contain multiple different cell populations derived from the host as well as the tumour cells. These cells include populations derived from the bone marrow (e.g. lymphocytes, macrophages/monocytes, granulocytes and dendritic cells), as well as cancer-associated fibroblasts and various stromal populations including the cells and stromal components comprising the vasculature (for an overview of the potential role of the various cell populations in the tumour microenvironment and how they may interact with radiation, see [Figure 1](#)).<sup>1</sup> Furthermore, it is now well established that owing to their genetic instability, the tumour cells themselves may consist of multiple clonal populations that reflect the evolution of the tumour and the ability of different genetic or epigenetic alterations to promote growth within the tumour mass. However, only a fraction of the tumour cells (the stem cells) may have long-term proliferative potential and the ability to regenerate the tumour. The microenvironment of the tumour cells plays a significant role in the tumour response to radiation treatment. Low levels of oxygen (hypoxia) caused by the poorly organized vasculature in tumours have long been known to affect radiation response.<sup>2,3</sup> However, other

aspects of the microenvironment also appear to play important roles. There are increasing numbers of reports implicating the potential role of radiation in enhancing immune activity against tumour cells.<sup>4,5</sup> There is also renewed interest in the potential role of radiation damage to the vasculature, in particular, its ability to recover following radiation treatment, so that it can support tumour regrowth. Blocking such recovery has been reported to increase the response of tumours to radiation treatment.<sup>6</sup> Radiation treatment can cause a significant influx of bone marrow-derived cell (BMDC) populations into both normal tissues and tumours.<sup>7</sup> Potential roles of such cells may include enhancing vascular recovery as well as modulating immune reactivity or possibly enhancing metastasis.<sup>8,9</sup> High levels of neutrophils in the circulation and the tumour have also been associated with poor treatment outcome in cancers following irradiation.<sup>10–12</sup> In this article, I will review some of the old literature concerning tumour response to radiation treatment and relate this to current concepts about the role of the microenvironment in tumour response to radiation treatment.

### RETROSPECTIVE

Prior to the development of *in vivo*–*in vitro* clonogenic assays for mammalian cells growing in culture, studies of the response of tumours to irradiation were largely conducted using growth delay or tumour cure assays in

Figure 1. Multiple cell populations in the tumour microenvironment can be affected by that environment and by irradiation. Reproduced from Barker et al<sup>1</sup> with permission from Nature Publishing Group.



rodents.<sup>13,14</sup> Many of these studies were conducted using transplantable tumours given single radiation doses or a few dose fractions. These studies generally established that fairly large doses of irradiation were required to cure such tumours, unless the tumour was grown in an animal that was not “immune-compatible” or the tumour was chemically induced, in which case, much lower doses could be curable indicating the potential role of the immune system.<sup>15,16</sup> These studies demonstrated that animals in which “immune-incompatible” tumours were grown and had been cured were largely resistant to a secondary transplant of that tumour, whereas this was not the case for tumours grown and cured in animals that were immune-compatible with the tumour involved (usually tumours which had arisen spontaneously in the inbred animal strain used for transplantation). These findings led to the view that chemically induced tumours were not very relevant models for assessing tumour response to radiation treatment and that such studies

were better conducted with spontaneously arising tumours grown or transplanted into immune-compatible inbred hosts, which were generally found to show very limited evidence of immune reactivity.<sup>17,18</sup>

### RADIATION RESPONSE OF TUMOUR CELLS

Following the development of *in vivo-in vitro* clonogenic assays and the development of cell lines derived directly from tumours, response of tumours focused on the radiosensitivity of the tumour cell population. Malignant cell populations in culture were found to have quite consistently shaped survival curves with a shoulder followed by a decline that could be reasonably well fitted by an exponential, although various different models have been proposed to fit such curves.<sup>19–22</sup> The current model of choice is the linear-quadratic model [ $S = \exp - (\alpha D + \beta D^2)$ ], and the survival data shown in Figure 2(b) are fitted with this model. In general terms, the alpha parameter defines the slope of

the initial part of the curve and the beta parameter defines the curvature of the curve. A derivation from this model has been widely used to compare response of tumours and normal tissues to different fractionated treatments, over a range of doses, based on estimates of the alpha/beta ratio.<sup>23</sup>

It should be noted that survival following radiation treatment is specifically defined in terms of the retention of proliferative capacity of the cells (*i.e.* as the ability of an individual cell to regenerate a colony of a certain size—usually 50+ cells) and does not relate to whether the cell actually dies and is lost from the population as a whole. This was consistent with observations that although some cells would undergo apoptosis following radiation treatment (depending to some extent on tissue type), many cells would often attempt cell division a few times before ultimately dying (of mitotic catastrophe) and being lost from the culture (or tumour) or undergoing senescence and ceasing proliferation. Thus, it was argued that the rate of regression of tumours following irradiation treatment largely reflected the underlying proliferative rate (fraction of cells in the proliferative cycle or the labelling index) of the tumour cell population. This in turn led to the view that the rate of regression of the tumour did not necessarily reflect the actual level of loss of cell survival due to the treatment and that, in tumour-regrowth assays, it was necessary to allow the tumour to regrow to larger than treatment size in order to determine the true response to the treatment in the context of surviving cells.<sup>24</sup>

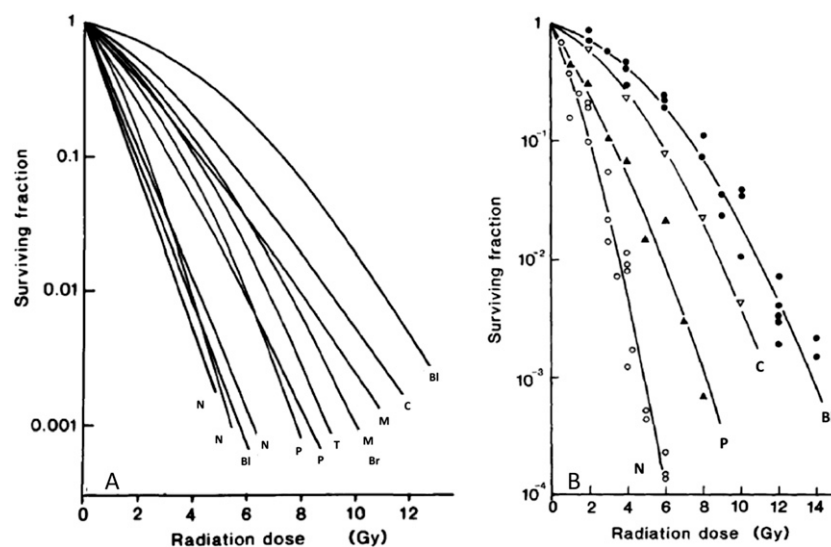
It was established that the shoulder of the survival curve reflected the ability of the cells to repair radiation damage<sup>25</sup> and the observed differences in survival curves for cells in different phases of the cell cycle were also largely thought to relate to different abilities of the cells in these various phases to repair radiation damage.<sup>26</sup> Subsequent studies have also indicated that at doses <0.5 Gy, some cell populations may have a “hypersensitivity” to irradiation, which is thought to relate to reduced repair capacity following these low doses, and that, at slightly

higher doses (>1 Gy), there is enhanced repair of this damage and that this may be related to cell cycle status.<sup>27,28</sup>

Repair of radiation damage associated with the shoulder of the survival curve has been modelled with various combinations of exponential functions but appears to be reasonably well fitted using a biexponential with rapid repair having an approximate half-time of about 0.3–0.5 h and slower repair with an approximate half-time of 4–5 h.<sup>29</sup> However, similar to the extent of repair, these half-times can be variable for different cells with values ranging by about a factor of 2 around these values and the longer repair times may be more relevant to late responding normal tissues than most tumours.<sup>30</sup> An important consequence of repair is that the efficacy of radiation treatment depends on the dose rate, particularly when this is below a value of about 0.5–1 Gy min<sup>-1</sup> with treatment generally becoming less effective below this value. This is true for both tumour cells and normal tissues and the extent of the effect depends on the repair capacity of the relevant cell populations. Thus, for cells with low repair capacity, such as bone-marrow-derived populations or tumours such as neuroblastoma, the effect is small, whereas for tissues or tumours whose cells have high repair capacity such the lung and gastrointestinal tract, it can be a large effect.<sup>31</sup> Although the instantaneous dose rate is important, for treatments using multiple beam protocols (such as intensity-modulated radiation therapy), the effective dose rate per treatment session also needs to be considered, and modelling suggests that treatments which exceed about 15–30 min may have reduced treatment efficacy.<sup>29,32,33</sup>

Studies of the radiation sensitivity of human tumour cell lines derived from different tumour types demonstrated that there were significant differences in the sensitivity of cells from different tumours (Figure 2). These effects were observed in relation to both the slopes and, particularly, the size of the shoulders of the survival curves for different cell lines, although

Figure 2. (a, b) Cell survival curves for various different human tumour cell lines treated with a single dose of radiation. (b) Data are fitted with the linear-quadratic model of cell survival. Br, breast; BI, bladder; C, cervix; M, melanoma; N, neuroblastoma; P, pancreas; T, teratoma. Modified from Steel<sup>148</sup> and Steel et al<sup>149</sup> with permission from Elsevier.



in no case was it observed that cells were absolutely resistant to radiation. The analysis of the survival curves of cells from different types of tumours led to the view that differences in the shoulder region of the survival curve (*i.e.* at a dose of 2 Gy) reflected the *in vivo* radioresponse of the different types of tumours in patients, given “standard” fractionated radiation treatment (*i.e.* ~2 Gy fractions), but that this was not generally true for the slope of the survival curve (Table 1).<sup>34,35</sup> This concept was followed up by a number of different groups, notably West et al,<sup>36,37</sup> who adapted *in vitro* assays to assess the radiation sensitivity of tumour cells derived directly from biopsies of cervical cancers in females due to undergo radiation treatment. The results of these studies indicated that the survival following a dose of 2 Gy correlated with the treatment outcome of the patients from whose tumour the cells were derived, indicating the importance of radiation sensitivity of the tumour cells for tumour response to irradiation (Figure 3). Subsequent studies reported similar effects for head and neck cancers, but other studies did not show significant effects.<sup>38</sup> Owing to the difficulty of performing the technique, the time necessary to obtain the result and the fact that for a significant fraction of patient’s tumours no data could be obtained, this approach to personalizing treatment based on the individual radiosensitivity of cancer cells in individual patients was not pursued further. However, the data are important both in the context of the studies of cancer stem cells (CSCs) (see Cancer Stem Cells section) and in relation to the increasing use of high dose treatment strategies [stereotactic body radiotherapy (SBRT)] for which response in the shoulder region of the survival curve is of limited relevance.

More recent approaches have involved using genetic analysis of multiple cell lines in attempts to identify genetic signatures which predict radiation sensitivity above and beyond the known effects of mutations in the ataxia telangiectasia mutated (*Atm*) and other DNA repair genes.<sup>39,40</sup> This approach has defined a variety of genes that may be involved in radiosensitivity of different tumour cell populations, but there are various limitations to such studies, notably the use of high throughput analyses of radiation response of the cells, which limits the

ability of the studies to examine genetic factors separately affecting the low-dose *vs* the high-dose region of the survival curve. Other concerns with such studies can relate to the potential selection bias associated with the growth of cells *in vitro* (if cell lines are used in the study) or the potential role of heterogeneity and multiple clones within tumours (if biopsies are used in the study). Studies to identify genetic factors affecting normal tissue response to irradiation are also under way based on analyses of patients who have experienced serious side effects of radiation treatment,<sup>41,42</sup> and these studies may also be informative concerning genetic factors affecting radiation response of specific tumour cell populations. To date such analyses have not translated into significant attempts to personalize radiation therapy in the clinic.

### ROLE OF HYPOXIA

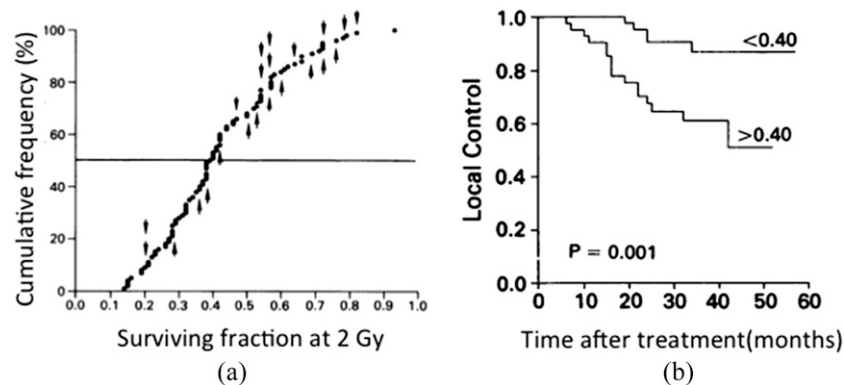
The further development of *in vivo/in vitro* assays to allow malignant cells taken directly from tumours to be grown in culture allowed studies of the radiosensitivity of malignant cells irradiated *in situ* in the tumour growing in a host animal. These studies confirmed earlier data from *in vivo* growth delay or tumour cure assays that tumours contained a fraction of viable cells at low oxygen levels (hypoxic) that were more resistant to irradiation than well-oxygenated cell populations (Figure 4).<sup>43,44</sup> These observations were a clear demonstration of the role of the microenvironment of the tumour cells on their response to irradiation, since it was established that resistance was directly due to the lack of oxygen during radiation treatment. Detailed studies demonstrated that the effect was largely due to direct interaction of oxygen with radiation-induced radicals on the DNA-preventing rapid chemical repair of the radical by antioxidant molecules such as sulphhydryls (–SH).<sup>45</sup> The level of oxygen required for an observed decrease in radiation response was less than about 10 mmHg, but full resistance was not observed until oxygen levels were below approximately 0.5 mmHg. Studies indicated that the levels of free sulphhydryls (*e.g.* glutathione, cysteine etc.) in the cells can modify this range; a factor that may be different for cells in tumours *vs* those growing in culture.<sup>46,47</sup> An important aspect of hypoxia in tumours was the finding that it was quite dynamic and that during the course of

Table 1. Values of the surviving fraction at 2 Gy for human tumour cell lines

	Tumour cell type	Number of lines	Mean survival at 2 Gy (range)
1.	Lymphoma Neuroblastoma Myeloma Small-cell lung cancer Medulloblastoma	14	0.20 (0.08–0.37)
2.	Breast cancer Squamous-cell cancer Pancreatic cancer Colorectal cancer Non-small-cell lung cancer	12	0.43 (0.14–0.75)
3.	Melanoma Osteosarcoma Glioblastoma Hypernephroma	25	0.52 (0.20–0.86)

Tumours are grouped 1–3 in approximate order of their likelihood of tumour control by radiation. Modified from Deacon et al.<sup>34</sup>

Figure 3. (a) Surviving fraction at 2 Gy (SF2 value) for primary cells derived from 88 different cervical cancers and (b) Kaplan-Meier plot of local control for those cancers given radiation treatment based on the SF2 value being above or below the median value (0.4). Reproduced from West et al<sup>36</sup> with permission from Nature Publishing Group.



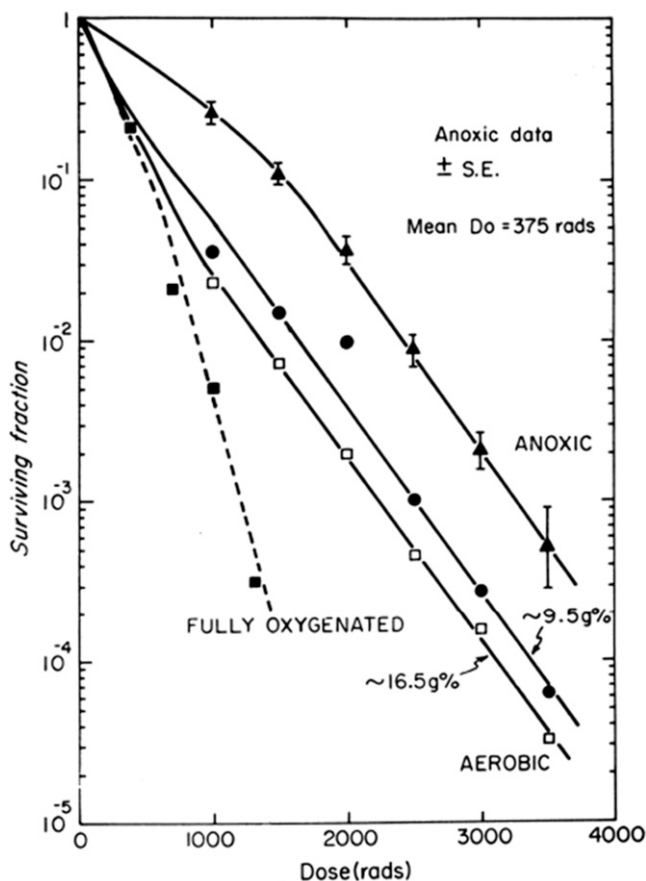
fractionated radiation treatment, hypoxic cells that had survived one fraction could become “reoxygenated” and hence become more sensitive to a subsequent fraction. This was a widely observed phenomenon in both transplanted and spontaneously arising tumours.<sup>48–50</sup> It was subsequently shown that tumours usually contain regions subject to fluctuating blood flow,<sup>51,52</sup> leading to the concept that tumour cells may be exposed to either chronic or acute (cycling) hypoxic environments and that observed reoxygenation could be partially explained by such cycling hypoxia. Chronic hypoxia is largely thought to occur as a result of oxygen diffusion limitations, but potentially, it may also result from the presence of unusually long vessels or slow blood flow when the oxygen capacity of the blood may be exhausted along the length of the vessel.<sup>53,54</sup>

Follow-up on the effect of hypoxia on tumour growth and response to treatment has continued to the present day as more and more knowledge has been accumulated about the effects of different levels of hypoxia on cellular metabolic function, and it is increasingly clear that other aspects of the effects of hypoxia may also play an important role in the response of tumours to treatment.<sup>55</sup> Relevant to radiation response are observations that the extent of repair of radiation damage in hypoxic cells depends on the length of their exposure to hypoxia both before and after irradiation, such that chronically hypoxic cells may have a lesser degree of resistance to radiation (due to reduced repair capacity) than acutely (cycling) hypoxic cells.<sup>56</sup> Early studies had also suggested such an effect using tumour cure assays.<sup>57</sup> Furthermore, cells may have limited ability to maintain viability long term under chronic hypoxia, depending on such factors as their ability to induce mechanisms of survival under low-energy conditions, such as autophagy.<sup>58</sup> Thus, some chronically hypoxic cells may not be able to induce tumour regrowth even if they could survive the initial radiation treatment. The possibility that hypoxic cells may die *in vivo* following doses of radiation but be able to survive if reoxygenated was proposed quite early but was quite variable in different studies.<sup>59–61</sup> Recent studies have suggested that this may be an important factor in the response of tumours to SBRT.<sup>62</sup> These observations are consistent with the concept that acutely hypoxic cells and/or the larger number of cells that are partially resistant to irradiation due to being at intermediate oxygen levels in tumours may in fact represent

a more important population of cells relevant to tumour control effected by standard fractionated radiation therapy rather than the most hypoxic cell populations.<sup>63</sup>

However, despite the overwhelming data that many tumours contain hypoxic cells and the findings that levels of hypoxia in various tumour types are predictive of treatment outcome, approaches to addressing this issue in radiation therapy treatment of patients have had only limited success. Initial studies using hyperbaric oxygen breathing or drugs which could specifically radiosensitize hypoxic cells showed some positive effects, and a meta-analysis of many of these early trials did show a significant gain overall.<sup>64</sup> However, a number of clinical studies testing the potential benefit of drugs that were directly cytotoxic to hypoxic cells have met with limited success when combined with radiation.<sup>65</sup> Partly, this may be due to the process of reoxygenation (and cycling hypoxia) occurring between treatment fractions, since pre-clinical studies did generally demonstrate that the degree of sensitization achieved with the radiosensitizer misonidazole declined to small values in the range of fraction sizes (1.5–2.5 Gy) generally used in radiation therapy (Figure 5).<sup>66</sup> However, another factor is likely to be the heterogeneity in the levels of hypoxia in different tumours. None of the studies of hypoxic radiosensitizers or hypoxic cytotoxins have involved measurements of levels of hypoxia in the tumours of all the patients included in the studies, thus it was impossible to identify if the patients with the most hypoxic tumours did in fact benefit from the drug treatment. This is particularly relevant since small subpopulations in these studies, in whose tumours’ hypoxia was measured, did demonstrate benefit in the most hypoxic tumours,<sup>67</sup> and a derived genetic signature associated with hypoxia-induced genes did show a correlation of the most hypoxic tumours with benefit from combination of a radiosensitizing drug (nimorazole) with radiation treatment.<sup>68</sup> The combination of robust techniques for imaging hypoxia prior to and during treatment with some form of genetic analysis would be consistent with current moves towards more personalized therapy. The supplementation of such genetic signatures with genes associated with the ability of cells to survive hypoxic stress or those that are associated with the environment of an hypoxic stem cell niche could potentially be beneficial but will need to be tested in future studies.<sup>69</sup>

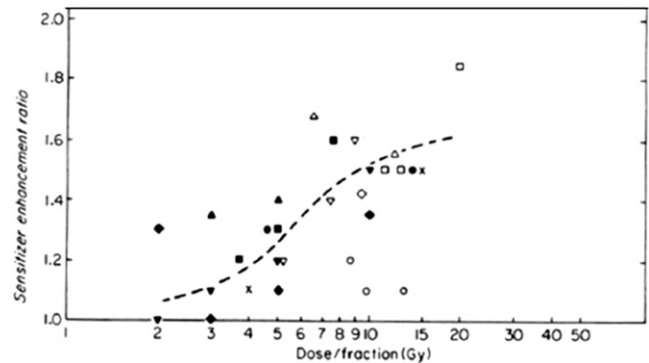
Figure 4. Tumour cell response to irradiation when cells are removed from the tumour for analysis of clonogenic capacity immediately after irradiation. The figure demonstrates: (1) the effect of making the tumour hypoxic immediately before irradiation (anoxic), (2) the effect of different levels of haemoglobin in the blood of mice on the fraction of hypoxic cells in tumours in air-breathing animals. Survival curve for fully oxic cells in tumours in air-breathing animals. Survival curve for fully oxic cells is shown for comparison. Reproduced from Hill et al<sup>44</sup> with permission from British Institute of Radiology. Do, slope of the survival curve; SE, standard error.



## CANCER STEM CELLS

Recent studies have highlighted the potential importance of stem cell populations in tumours—CSCs.<sup>70,71</sup> Such cells (CSCs) are argued to be ultimately responsible for maintaining tumour growth and for recurrence following therapy. They thus represent the critical population for predicting treatment outcome, and their number and radiosensitivity are important for tumour control by radiation therapy. The numbers of CSCs in tumours have been reported to vary widely, and it has been demonstrated in animal models that the number of (putative) stem cells correlates with the single radiation dose required for tumour control (Figure 6).<sup>72,73</sup> Similar results have been reported for experimental studies in animal models using fractionated radiation treatment<sup>74</sup> and the expression of the stem-cell-related marker CD44 has been reported to correlate with local control in early laryngeal cancers treated with radiation.<sup>75</sup> Interestingly, in the studies of West et al<sup>76</sup> discussed above, the plating efficiency (PE) of the cells from cervical cancers was not found to be as good a predictor of patient outcome as the SF2 value

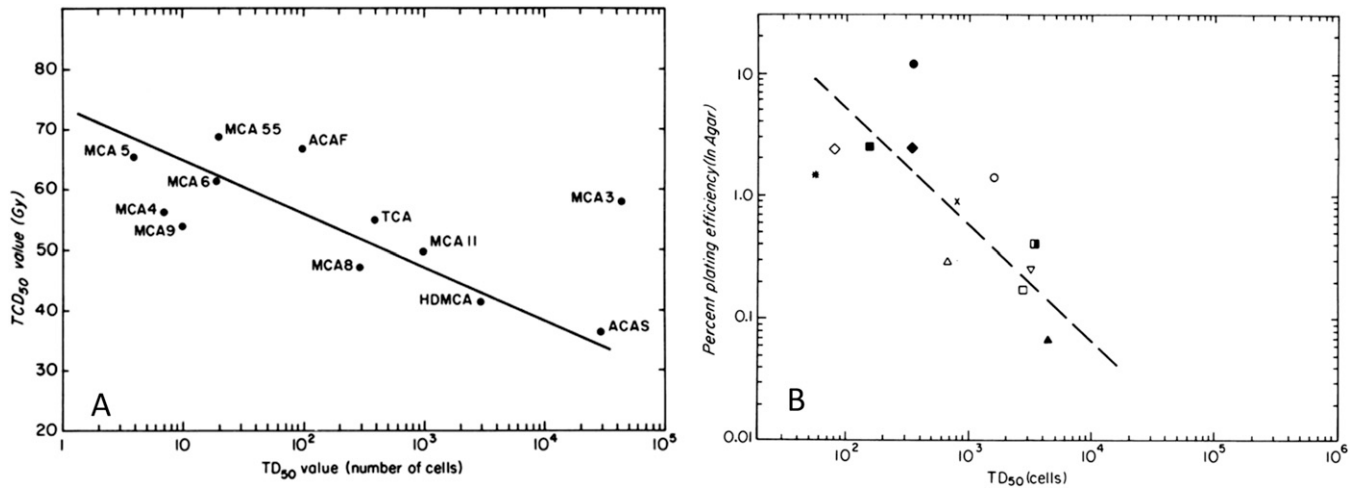
Figure 5. The sensitizer enhancement ratio obtained when different animal tumours were treated with fractionated radiation doses in the presence or absence of misonidazole is plotted as a function of the dose per fraction. The different symbols represent data from different experiments. The broken line is drawn to represent the trend of the data. Modified from Hill<sup>66</sup> with permission from Elsevier.



determined *in vitro*. However, *in vitro* assays may not reflect well the long-term potential for proliferation required of CSCs. In studies of cells derived from spontaneous mouse mammary tumours, there was a correlation of PE *in vitro* with the number of cells required to be injected to successfully achieve 50% transplant of the tumours (TD50 value), which is regarded as the gold standard for assessing CSCs, but the *in vitro* PE was greater than a factor of 10 higher than the *in vivo* TD50 value (Figure 7).<sup>72</sup> This is consistent with the data for murine bone marrow, for which there are cells that can form colonies *in vitro* as well as cells that can be classified as intermediate-term and long-term stem cells *in vivo*.<sup>77,78</sup>

There are several data sets which support a higher radioresistance of CSCs than their progenitor cells in tumours.<sup>73,79</sup> In extensive experiments, an increase of the *ex vivo* fraction of CD133<sup>+</sup> cells, confirmed as CSC by transplantation assays, was observed after *in vivo* irradiation of glioma xenografts, and CD133<sup>+</sup> cells were found to be more radiation resistant *in vitro* (Figure 7).<sup>80</sup> There was preferential activation of DNA damage checkpoints in CD133<sup>+</sup> vs CD133<sup>-</sup> cells. Another resistance mechanism may be related to higher levels of antioxidant molecules to deal with reactive oxygen species (ROS), a critical mediator of radiation damage in cells.<sup>81</sup> Compared with progenitor cells, breast CSCs have been shown *in vitro* to contain a lower level of ROS with higher expression of genes involved in ROS scavenging. Moreover, the initially higher post-irradiation clonogenic cell survival of breast CSC can be altered by pharmacological modulation of the ROS levels. However, a higher intrinsic radioresistance of CSC cannot be regarded as a general phenomenon, since heterogeneity seems to exist between individual tumours of the same histology.<sup>82</sup> A link between hypoxia and putative stem cells has also been shown by an increase in the fraction of CD133<sup>+</sup> cells in brain tumour cells exposed to hypoxia *in vitro*,<sup>83,84</sup> and the preferential expression of hypoxia induced factor (HIF)-2 $\alpha$ - and HIF-regulated genes in glioma stem cells.<sup>85</sup> Specific microenvironmental factors such as cell-cell interactions and genetically regulated cellular signals may also be important determinants for stem cell maintenance and

Figure 6. Relationship between tumour “stem-like” cells and tumour control for a group of first passage mouse mammary tumours in C3H mice. (a) The relationship between the single radiation dose given under hypoxic conditions required to cure 50% of the tumours (TCD<sub>50</sub> value) and the number of cells required to be injected to generate growth of tumours in 50% of injection sites (TD<sub>50</sub> value). The slope of the line is consistent with the radiation sensitivity of hypoxic cells. (b) The relationship between the *in vitro* plating efficiency and the TD<sub>50</sub> value for the same group of tumours. Modified from Hill and Milas<sup>72</sup> with permission from Elsevier.

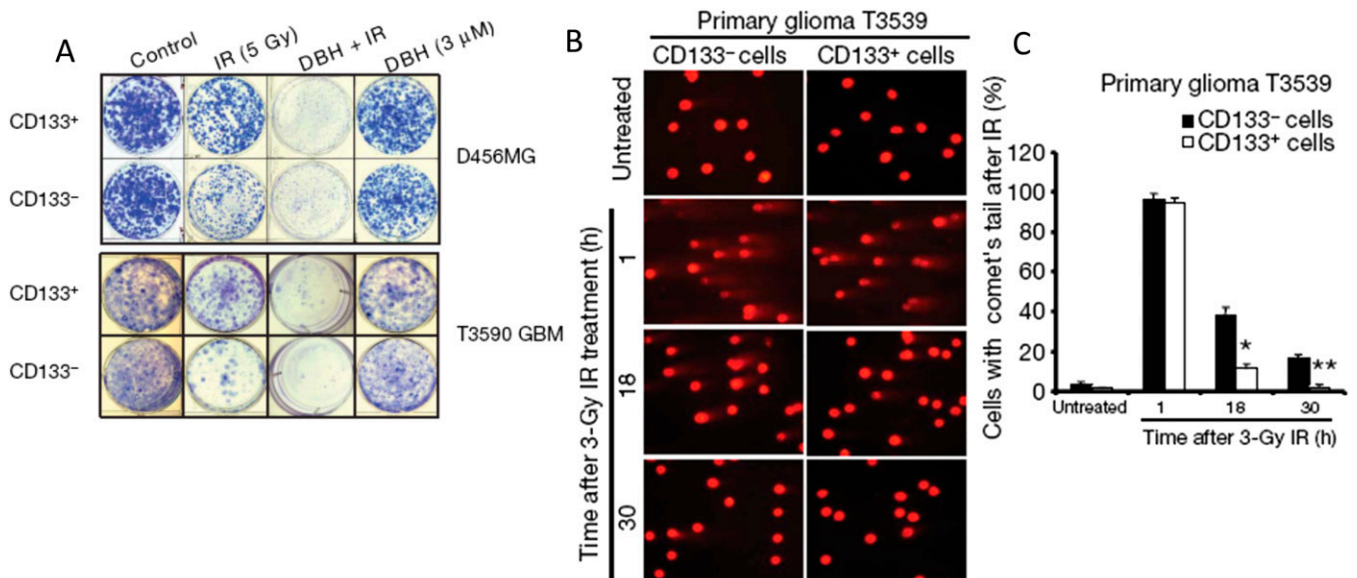


survival. Different kinds of “niches” have been described that contain a higher fraction of CSCs or maintain the stem-like phenotype of tumour cells, including a hypoxic niche.<sup>86,87</sup> A hypoxic niche for CSCs would be expected to affect their relative radiosensitivity, but if the level of hypoxia is consistent with increased levels of HIF-1 $\alpha$  and HIF-2 $\alpha$  (approximately <10–20 mmHg) rather than the levels required for full hypoxia-induced radioresistance (<1–5 mmHg),<sup>88</sup> it may only cause a small increase in radioresistance. However, as noted above, oxygen sensitization relates to a competition between oxygen and free sulfhydryls to interact with the radiation-induced radical sites on damaged DNA,<sup>47</sup> thus higher levels of SH-

containing antioxidant molecules such as glutathione or cysteine in CSCs might enhance the protection associated with intermediate levels of hypoxia.

Another aspect of the issue of radiation sensitivity of CSCs is the finding that allowing tumour cells to grow in three-dimensional cultures or “spheroids” can enhance the fraction of them which express the phenotype of stem cells.<sup>89,90</sup> This is of interest in the context that such growth has also been noted to modify the radiation sensitivity of cells (independent of oxygen effects). Early work by Durand and Sutherland<sup>91</sup> found that cells from spheroids had an increased shoulder on their radiation survival

Figure 7. Reduced radiation sensitivity of stem cells. (a) Plating of CD133<sup>+</sup> (stem cells) or CD133<sup>-</sup> (progenitor cells) glioblastoma cells irradiated or not with 5 Gy and treated or not with debromohymenialdisine (DBH), a CHK1/2 inhibitor. The CD133<sup>+</sup> cells show higher plating after 5 Gy than the CD133<sup>-</sup> cells. (b) Alkaline comet assays of CD133<sup>+</sup> or CD133<sup>-</sup> cells from one of the cell lines at various times after irradiation (IR) with 3 Gy. (c) The quantification of the number of cells retaining comet tails (*i.e.* those that have not completed DNA repair) at various times after irradiation. Reproduced from Bao et al<sup>80</sup> with permission from Nature Publishing Group.



curve and that this reflected increased repair capacity. This was demonstrated to decay away over a period of about 24 h when the cells were separated into single cells.<sup>92</sup> A similar effect was observed *in vivo* with cells growing as small colonies in the lung after they were removed and assayed for radiosensitivity as a single-cell suspension (Figure 8).<sup>93</sup> More recently, increased radiation resistance has been reported for cells growing in three-dimensional culture or on various extracellular matrix substrates. This resistance has been related to the changed expression of certain cell surface molecules such as integrins and focal adhesion genes, which are involved in cell–cell and cell–matrix interactions.<sup>94–97</sup> An important difference here may be the usual absence of serum in the medium used for stem cell studies in spheroids, but it is unclear whether the change in radiation sensitivity associated with growth in spheroids is directly related to the “stemness” of the cells or is a parallel phenomenon.

A further concern with studies of CSCs is the increasing evidence for plasticity of the CSC phenotype and the concept that early progenitor cells may be able to regain stem cell properties, thereby increasing the effective number of CSCs in the tumour.<sup>98</sup> Particularly important in the context of radiation treatment is that back differentiation of progenitor cells into stem cells may be induced by radiation, although the extent of this phenomenon is currently uncertain.<sup>99–102</sup> However, comparisons of the radiation dose required to cure xenografted tumour cell lines in immune-deprived mice using single doses *vs* fractionated treatments have given a reasonably good correlation, suggesting that this effect may be limited.<sup>74</sup> Further studies are needed in this area, particularly with tumours directly from patients and early patient-derived xenograft (PDX) models. Studies of murine bone marrow stem cells have demonstrated stem cell populations with intermediate-term or long-term ability to repopulate the marrow,<sup>78</sup> but little is known about whether such populations may exist in tumours. The potential

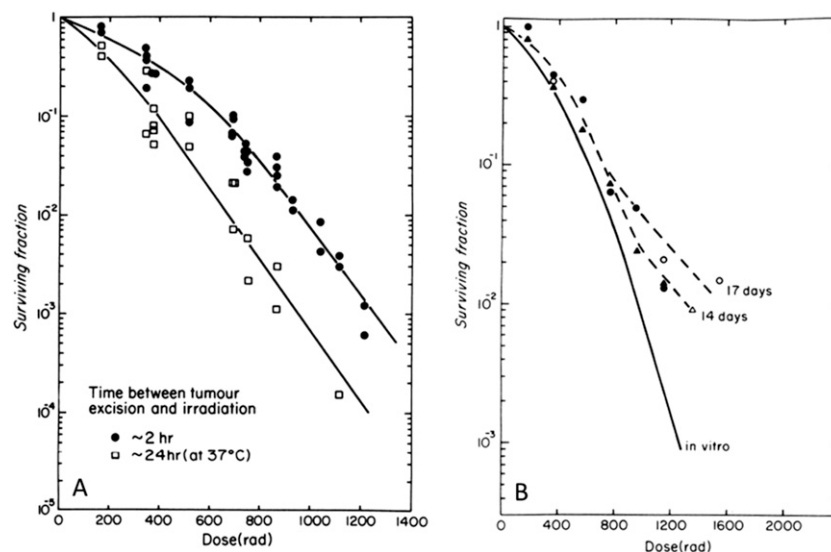
role of genetic heterogeneity and genetic instability also needs to be considered, since this could relate to selection of radiation-resistant cell populations following treatment.

## RADIATION-INDUCED DAMAGE TO VASCULATURE

The potential role of radiation damage to vasculature in relation to tumour response was presaged by early studies examining what was called the “tumour bed effect”. The essence of these observations was that transplanting a tumour into a site in an animal that had been previously irradiated resulted in a requirement for larger numbers of cells for transplantation and slower growth of the subsequent tumour than was observed when the same type and number of tumour cells were transplanted into a non-irradiated site.<sup>103</sup> These observations were also consistent with early reports that tumours regrowing after large doses of irradiation had an initial phase of slower growth before increasing<sup>14</sup> or that regrowing tumours following radiation treatments often grow more slowly than untreated tumours. This latter observation led to recommendations that such a difference in growth rate could be built into analysis of growth delay studies by calculating the delay in terms of the number of tumour growth doublings rather than the absolute delay time. Importantly, these effects were largely observed following quite large doses of irradiation (10–20 Gy+) and limited tumour-bed effects were observed following lower doses. These observations suggest that vascular damage could impact the ability of tumours to regrow after irradiation and are consistent with the more recent data on the effect of radiation-induced apoptosis in endothelial cells on tumour response.<sup>104</sup> Doses in excess of about 8–10 Gy are needed to induce this effect.<sup>105</sup>

The concept that damage to tumour vasculature affects tumour response was tested in a study that examined the single doses of

Figure 8. Increased shoulder on the survival curve for cells growing in contact *in vivo*. (a) The effect of holding cells obtained from an intramuscular tumour (KHT sarcoma) *in vitro* for 2 or 24 h before irradiation. The data points are from analyses of cells from individual groups of tumours. (b) Results for irradiation of the cells as small colonies growing in the lung compared with a survival curve for irradiation of the same cells growing *in vitro*. The tails on these curves show the development of hypoxia in these small lung colonies. The individual points represent analysis of different groups of lungs. Reproduced from Hill et al.<sup>93</sup> with permission from Radiation Research.



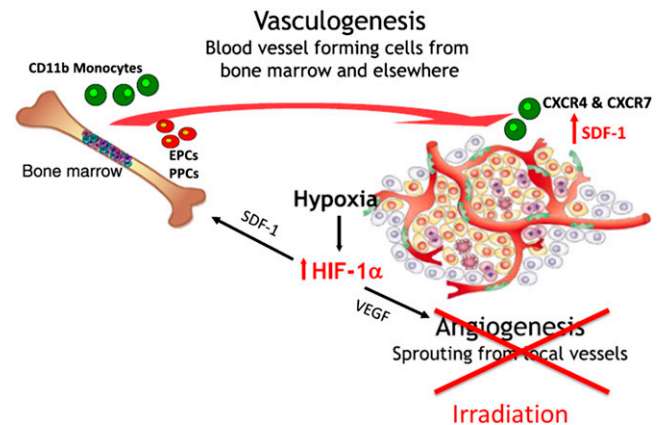


radiation required to control a series of transplantable tumours growing in either nude or severe combined immunodeficiency (SCID) mice.<sup>106</sup> Since the *scid* mutation makes the cells of SCID mice more radiation sensitive, it was argued that the vasculature of tumours growing in these mice should be more sensitive than those growing in nude mice and, consequently, that tumours growing in SCID mice should require a lower dose for cure than those growing in nude mice. This was not found to be the case, thus it was concluded that damage to vasculature did not play a significant role in tumour response to irradiation. Similar results have also been reported recently for cure of a rodent tumour model in which endothelial cells were sensitized by a mutation in the *Atm* gene (which affects DNA repair).<sup>107</sup> However, in this study, it was found that radiation-induced growth delay was enhanced in the tumours with the more sensitive endothelial cells, suggesting that increased damage to vasculature could affect the ability of the tumour to regrow over time, although not the dose required for cure. Various possible mechanisms of vascular repair that could occur over time include the presence of circulating endothelial precursor cells, observations that certain tumour cells can transdifferentiate into endothelial cells and the findings that there is an influx of BMDC populations into irradiated tissue that can potentially help to support the vasculature during recovery.<sup>7</sup> Studies using a window-chamber model have delineated the effects of large doses of radiation on depleting vascular function, particularly the microvasculature, in tumours and demonstrated the recovery of function a number of days later.<sup>108–110</sup>

Following the work of Folkman<sup>111</sup> on the essential role of angiogenesis in tumour growth, there has been the development of agents that target vasculature, both in terms of blocking angiogenesis and by direct disruption of tumour vasculature.<sup>112</sup> A number of antiangiogenic agents are approved for clinical application,<sup>113</sup> and some of these agents have been combined with radiation treatment. To date, these studies have shown quite limited benefits,<sup>114</sup> and it is hypothesized that this may be due to multiple angiogenesis pathways and/or differential ways in which tumour vasculature may recover following radiation treatment, primarily by angiogenesis or vasculogenesis. A major difference between these two pathways is new vessels sprouting from existing vessels (angiogenesis) *vs* the influx of cells from other parts of the body or bone marrow, which can build or rebuild vessels (vasculogenesis).<sup>115</sup> Various vascular disrupting agents are also in clinical development,<sup>116</sup> and pre-clinical studies have suggested that these agents have potential to be combined with radiation, but apart from anecdotal observations in early stage trials, it is unclear if these will prove to be any more beneficial than antiangiogenic agents.

Recently, blocking vasculogenesis has been postulated to be important for exploiting the damaging effects of radiation on the vasculature.<sup>6</sup> This has arisen partly from the view that angiogenesis is largely an in-field phenomenon, and consequently, since it should be impaired by the radiation treatment, it is likely vasculogenesis that is responsible for vascular repair following radiation treatment (Figure 9). This is consistent with the influx of BMDC that occurs in irradiated tissues and the various findings suggesting that BMDC may play a role in alleviating

Figure 9. Model for repair of vascular damage following irradiation. Inactivation of angiogenesis by a large dose of irradiation causes the tumour vasculature to rely on vasculogenesis for repair prior to tumour regrowth. Modified from Brown<sup>6</sup> with permission from British Institute of Radiology.

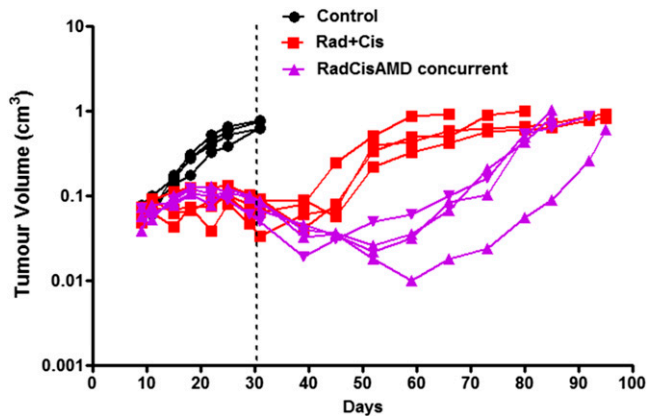


vascular damage. Such cells have been observed to associate with the outside of vessels in irradiated tissues in a manner similar to pericytes, and it is been hypothesized that this helps to maintain the structure of the vessels.<sup>7</sup> In fact it has been recently reported that mesenchymal stem cells from the bone marrow can differentiate and act as progenitors of pericytes (PPCs) in irradiated tumours.<sup>117</sup> Together with the presence of endothelial precursor cells (EPCs) in the circulation (whether these cells derive from the marrow is controversial), this influx of cells may help to stabilize and repair irradiated vessels. The influx of cells from the marrow has been demonstrated to be related to the production of various chemokines including C-X-C motif chemokine 12 [CXCL12; also known as stromal cell derived factor 1 (SDF-1)] and blocking the interaction of this chemokine with its receptors C-X-C chemokine receptor type 4 (CXCR4) or C-X-C receptor type 7 (CXCR7) has been reported to enhance tumour (glioblastoma) response to irradiation.<sup>6</sup> We have obtained similar results using ME180 cervical cancer xenografts growing orthotopically in mice, where it was found that blocking the interaction of CXCR4 with CXCL12 using AMD3100 (Plerixafor) increased the growth delay induced in the tumours by radiation plus cisplatin treatment (Figure 10). Plerixafor is a clinically available drug that helps to mobilize bone marrow stem cells into the circulation. Similar increases in tumour radiation response have also been obtained by blocking HIF-1/2a expression, which is one factor that can cause the upregulation of CXCL12 levels in irradiated tumours.<sup>118</sup>

## OTHER AREAS OF CURRENT INTEREST

One area that is exciting particular current interest is the combination of radiation therapy with immunotherapy. Attempts to enhance the immune reactivity of tumours have been investigated for many years in experimental models,<sup>119</sup> at least in part due to the occasional observations of abscopal effects of radiation in the clinic.<sup>120</sup> This work also built initially on studies with chemically induced tumours, which showed evidence of an ability of the immune system to act against such tumours. Our improved understanding of the evolution of the interaction of

Figure 10. Tumour response curves for an orthotopic cervix Ca xenograft (ME180) treated with fractionated irradiation ( $15 \times 2$  Gy given over 3 weeks) + cisplatin ( $4 \text{ mg kg}^{-1}$  given at the beginning of every week) (Rad + Cis) or Rad + Cis + AMD 3100 (Plerixafor) given throughout the course of treatment by infusion pump.



the immune system with malignant cell populations with the concept of “immunoeediting” in three phases—elimination, equilibrium and escape—during tumour development and growth has led to a variety of different approaches, including inhibition of *T*-regulatory cell activity, cytokine-based enhancement of *T*-cell activity, myeloid cell activators, checkpoint blockade and various therapeutic vaccines.<sup>5,121,122</sup> It is proposed that increased exposure of immune effector cells to tumour antigens due to the death of tumour cells following radiation treatment has the potential to enhance immune reactivity. A number of pre-clinical studies have reported improved response of radiation-treated tumours and in some cases of metastatic lesions not given radiation treatment.<sup>16</sup> The recent success of the use of immune checkpoint blockade in certain human cancers has raised hopes that such an approach may be further enhanced by radiation therapy and a number of clinical trials are under way to investigate various aspects of this idea.<sup>122,123</sup> In the context of radiation treatment, studies of abscopal effects suggest that the size of the radiation dose may be important for such effects and that standard fractionation schedules (2 Gy) may be less effective than larger doses per fraction (6–8 Gy) and that these may be superior to bigger single doses (20 Gy). The early data also suggest that giving checkpoint inhibitors concurrently with radiation is more effective than giving them after the end of the radiation treatment, but further studies are needed to enhance our understanding in these areas.<sup>124,125</sup> A separate but related issue is the induction of bystander effects in which irradiated cells may produce molecules that can induce damage in closely located cells that are not directly irradiated.<sup>126</sup> The role of such effects in standard radiation therapy is currently unclear, but they may play a role in radionuclide therapy.<sup>127</sup>

Another area of current interest is the use of SBRT treatments involving one or a few large fractions. Such treatments have largely been made possible by the improved ability to target radiation treatment and have met with significant success particularly in treatment of small lung cancer lesions.<sup>128,129</sup> The success of such treatments is at least partially due to the fact that

much larger effective doses (based on linear-quadratic modelling) can be delivered than was previously possible, without causing serious normal tissue side effects, and it is unclear if other factors play a significantly different role than for standard fractionation.<sup>130</sup> One possible factor of importance may be the role of hypoxia since pre-clinical studies have suggested that the extent of reoxygenation is related to the degree of fractionation as noted above. Whether SBRT schedules may be more appropriate for combination with hypoxia cell sensitizers or hypoxic cytotoxins remains to be determined. As noted above, any such studies will need to consider the level of hypoxia in the tumours and select suitable patients based on measurements of this level.

## FUTURE STUDIES

The recent decision by the National Cancer Institute/National Institutes of Health to retire the NCI60 panel of cell lines, long used to test new drugs, has highlighted concerns that drug testing using long-term cell lines has limited predictability for efficacy of such drugs in clinical application.<sup>131–133</sup> These articles have argued that evaluating new targets using established cell lines is limited by the often poor correlation between responsiveness observed in cell lines *vs* that elicited in the patient. It is argued that PDXs generated from fresh tumour specimens are likely to recapitulate better the diversity of cancers and are more reflective of the histopathology and properties of the original tumour. There is also increasing evidence that PDXs can recapitulate treatment responses of individual parental tumours.<sup>134,135</sup> Although these concerns were focused on drug response, rather than radiation response, it is very likely that they are relevant to studies of radiation effects in tumours.<sup>136</sup> This may be particularly so in modern radio(chemo)therapy with the widespread use of drugs in combination with radiation. These issues may also be a concern for studies trying to identify genetic changes in which large numbers of cancer cell lines are examined for correlations between genetic or epigenetic abnormalities associated with radiation response.<sup>40</sup> The findings of such studies will need careful follow-up in appropriate models before they can be used for personalizing radiation treatment strategies. Recent studies in primary tumours have, however, identified genomic instability as a driver of disease aggressiveness and treatment failure in both surgical and radiation treatment of prostate cancer.<sup>137–139</sup> Furthermore, it appears that patient-specific measurements of tumour hypoxia and genomic instability provide complementary information about clinical outcome, with hypoxia being more or less important in some genomic subgroups than in others,<sup>138</sup> emphasizing the role of the tumour microenvironment.

A further issue concerning suitable tumours for study relates to the site of transplantation of the tumours. Subcutaneous or intramuscular transplantation of tumours on a body extremity has long been the preferred site for experimental radiation studies in rodents due to simplicity and easy of access for treatment without excessive normal tissue exposure; however, it may not be the optimum site. Increasingly, orthotopic transplantation is preferred since tumours growing subcutaneously have clearly different properties such as reduced metastases, increased encapsulation and, in many cases, increased levels of necrosis as they grow, suggesting differences in vascular

function.<sup>140</sup> Modern small animal irradiators, which provide small defined focused beams and an ability to target the tumour from different angles have reduced concerns about normal tissue exposure and have largely obviated the need to use subcutaneous transplantation sites.<sup>141–144</sup> These current technologies are allowing investigation of treatment strategies not previously possible in small animals. They provide opportunities to test different approaches with the attending possible differences in the critical parameters affecting tumour treatment response, particularly those related with the microenvironment. These technologies also make it easier to address one downside to using PDX model, *i.e.* the need to use immune-deprived animals, which limits the study of any immune interactions with the radiation treatment. The availability of many genetically modified mouse models of different cancers or other spontaneously arising rodent tumours can help to address this concern, since they can be more easily treated in the site of origin, either as the primary tumour or following orthotopic transplantation in the same context as PDX models.<sup>136,145,146</sup>

In summary, the emerging evidence of the multiple factors that may affect radiation response impacts the ability to select suitable tumours for personalized changes in radiation therapy treatment. Such selection needs to encompass the microenvironmental factors associated with tumour growth and response to treatment, as well as specific aspects of the radiation sensitivity of the tumour (stem) cells, related to aetiological, genomic

and epigenomic factors. The increasing long-term survival of patients with cancer has prompted greater concern about the side effects of radiation/chemotherapy treatments raising the issue of any associated changes on normal tissue response, since these are a critical component of the therapeutic ratio. Such issues may be partially addressed by improvements in the accuracy of precision radiotherapy and particularly the use of particle therapy (currently protons or carbon ions), which has the potential to reduce the volume of normal tissue exposed as well as exploiting the higher relative biological effectiveness associated with these radiations.<sup>147</sup> However, parallel studies of critical normal tissue responses will remain necessary as new drugs are introduced in combination with radiation treatment as part of personalized therapy.

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## CONFLICTS OF INTEREST

The author is part of a team involved in the development of a small animal irradiator (XRad 225Cx) and receives a royalty from the company (Precision X-ray) marketing the device.

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