Non-Targeted Radiation Effects in Radiotherapy

Roles of Radiation-Induced Genomic Instability and of the Bystander Effect in Cancer Cure by Radiotherapy

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Local tumour control by radiotherapy requires the complete sterilization of all tumour 'stem' cells in the tumour volume. Neither bystander effect nor radiation-induced genomic instability is able to contribute substantially to the probability of local tumour control of the primary cancer by radiotherapy. However, the progeny of these surviving tumour 'stem' cells are likely to suffer from radiation-induced genomic instability, which results in the persistent appearance of non-stem cells, i.e. a reduced probability of self-maintenance. This results in a slower growth rate of the recurrent tumour, a reduced stem-cell fraction and, as a consequence, an increased radiosensitivity of the recurrent tumour. In some recurrent tumours, particularly those that develop very late and grow very slowly, radiosensitivity may be further increased by increased intrinsic radiosensitivity, which could be related to the as yet poorly defined phenotype of 'small colony formation'.

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TUMOUR STEM CELLS AND TUMOUR CURE

It is a fact that radiotherapy can cause local tumour control. It is also a fact that irradiation causes the sterilization of clonogenic tumour cells in vitro and in vivo. Yet it is an interpretation that local tumour control is caused by the complete sterilization of all clonogenic tumour cells, also called tumour 'stem' cells, down to the last one, and that a single tumour 'stem' cell could be the origin of a tumour recurrence (1).

This interpretation is based on experiments in which spontaneous mouse tumours were transplanted into highly inbred isogeneic mice. The primary evidence for the interpretation comes from experiments conducted by Hewitt (2). When increasing numbers of isolated tumour cells were transplanted into mice, the probability of a new tumour growing in the recipients increased following a Poisson function. This led to the interpretation that a single cell could be the origin of a transplanted tumour. If the TD-50 was larger than one cell, and there could be several thousands, but the relationship between inoculum size and take rate still followed a Poisson function, it would mean that in such a tumour there would be only one stem cell among thousands of non-stem cells. When the tumour cells were irradiated before transplantation, more cells were needed for successful transplantation; however, the shape of the curve still followed a Poisson distribution, suggesting the interpretation that, again, a single cell that survived irradiation, while the others were sterilized, was the origin of the successful tumour transplant. In plotting the negative logarithm of the number of tumour cells for a 50% success rate against radiation dose, Hewitt (3), and later Berry (4) and several others, produced dose–effect curves that resembled those published a few years earlier by Puck for colony forming cells in vitro (5). This was interpreted by Gray (6) as indicating that both effects, i.e. loss of clonogenicity of irradiated single cells and local tumour control after radiotherapy, represented different manifestations of the same radiobiological fact, and that local tumour control depended only on the all-or-nothing effect of sterilization of all tumour clonogens/tumour stem cells. The most convincing experimental evidence supporting this interpretation in solid tumours in mice was published by Suit in 1965 (7). Since then, the all-or-nothing effect of tumour control or tumour recurrence has been based on a presumed all-or-nothing effect on tumour clonogen sterilization, which can be studied in vitro following the methods first described by Puck 45 years ago (5).

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NON-TARGETED, NON-QUANTAL RADIATION EFFECTS IN CLONOGENIC CELLS

Yet the experimental facts described by Puck (5) do not support the interpretation that the radiation effect on cells in vitro was indeed an all-or-nothing effect. The criterion of 50 progeny cells within 2 weeks as proof of clonogenic survival is fairly arbitrary. Those cells which do not succeed in producing 50 progeny in 2 weeks are still able to proliferate for some time and produce progeny. On the other hand, those that succeed in producing these 50 cells are by no means unharmed: their proliferative efficiency is seriously compromised, as was shown in Puck's first publication (5).

Of course, this has all been known for a long time, and discussed and dismissed as irrelevant for cancer cure by radiotherapy. Yet recent experiments examining this problem in more detail are forcing us to reconsider those old arguments. There are new experimental facts, mostly in vitro but also some in vivo, that challenge the old paradigm. They have been termed the bystander effect (8) and radiation-induced genomic instability (9).

The *bystander effect* describes the experimental fact that a cell which has not been hit directly, but that is close by, may show signs of damage. This effect has been particularly well demonstrated in experiments using the Gray Laboratory microbeam facility (10). Even if only one cell in the centre of a field within a non-confluent monolayer of fibroblasts received 5 protons targeted to the nucleus, many cells up to a few hundred micrometers away develop signs of apoptosis. If the bystander effects worked in tumours during radiotherapy, the basic assumption that we need to sterilize all clonogens directly by radiation hits to each individual cell (or by secondary, vascular mediated mechanisms of starvation) is not correct. This would have consequences for our understanding of how radiotherapy works.

Radiation-induced genomic instability describes the experimental fact that in the progeny of surviving irradiated cells the rate of newly arising cellular damage is persistently elevated (9). These signs of damage are stable or unstable chromosome aberrations, cell death, or decreased clonogenicity (11).

The dose and time dependence of the various manifestations of radiation-induced genomic instability vary widely between different cell lines in vitro, but they also occur in vivo; they have even be demonstrated in the developing embryo (12). The persistent decrease in colony forming ability appears to be related to the de-novo appearance of unstable chromosome aberrations (13), which suggests that, in surviving irradiated mammalian cells, a persistent increase in clastogenic activity may be transferred from cell generation to cell generation. Yet the target for the induction of this effect is not related to radiation damage to the nuclear DNA (9, 14). There is recent evidence that mitochondrial damage with persistently increased oxidative stress may play a key role in this consequence of irradiation in mammalian cells (10).

The whole concept of clonogenic survival was thrown into doubt when Mothersill et al. (15) demonstrated that the progeny of irradiated surviving cells were less clonogenic than the original clonogens before irradiation. They suggested that, in order to derive the true surviving fraction of clonogens after irradiation, the fraction of cells that produced colonies in the traditional Puck method should be multiplied by the colony forming efficiency of the progeny. If this was correct, all quantitative assessments of the relationship between local tumour control and surviving fraction would be wrong by orders of magnitude. However, the recommendation to multiply the surviving fraction by the residual colony forming efficiency of the progeny should not be applied to the cellular interpretation of radiation oncology. The concept that a surviving clonogenic cell has unlimited proliferative capacity, and is therefore able to grow into a recurrent tumour, does not mean that the cell is free from residual damage and that it will grow as fast as the same cell before irradiation. In considering the role of radiation-induced genomic instability for the cure of cancer, we do not need to consider the residual surviving fraction, as this is unrelated to the number of cells with unlimited proliferative potential. However, we have to discuss the possible effects of the reduced colony-forming efficiency of those cells that grow into the recurrent tumour, or which regenerate irradiated normal tissues (16). Other potential consequences of radiation-induced genomic instability in the recurrent tumour might be an increased rate of mutations, some of which might change the sensitivity of cells towards some chemotherapeutic agents (17).

ROLE OF THE BYSTANDER EFFECT IN TUMOUR CURE BY IRRADIATION

Whereas in external beam radiotherapy few radiobiologists doubted seriously that each tumour 'stem' cell had to be destroyed, be it directly by hitting a crucial target in its nucleus or, in some cases, by vascular effects such as those from hyperthermia or photodynamic therapy or by additive cytotoxicity in combined radio-chemotherapy, it has been proposed that in situations of very inhomogeneous irradiation of tumour cell populations, which is common in the therapeutic application of unsealed radionuclides (either incorporated directly or attached to some tumour-specific carriers), the negative effects of inhomogeneity would be corrected by bystander effects. It was assumed that cells that are lethally affected would send out death signals to neighbouring, bystanding cells that are not directly affected. This sounded like wishful thinking; however, quite recently a paper has appeared (18) claiming to have demonstrated exactly such a dramatic bystander effect in a very simple in vitro model.

Chinese hamster cells were labelled with high activity tritiated thymidine, mixed with unlabelled cells and then converted into multicellular clusters of varying proportions of unlabelled cells and cells which carried sufficient radioactivity to sterilize them. The cluster was maintained for 3 days in the refrigerator, then dissociated into single cell suspensions, and the cells assessed for colony survival. In line with expectations, the surviving fraction decreased with increasing activity if all cells were labelled with suicidal amounts of tritiated thymidine. However, if only 50% of the cells were labelled, one would expect a sharp decrease to 50% surviving fraction and then a plateau. In contrast to expectations, a biphasic dose–response curve was observed. After the surviving fraction decreased to 50% as expected, it decreased further with further increasing dose (albeit less steeply) to very low surviving fractions This experimental result was interpreted by the authors as evidence of a pronounced bystander effect which could be exploited in therapeutic nuclear medicine using anti-tumour antibodies as carriers for radioactivity. The results of these experiments, however, are at odds with a large number of older studies investigating the effect of radiation-sterilized tumour cells on the clonogenicity of tumour cells in vitro and in vivo. Some of the earliest were performed by Hewitt (3). All published experiments have demonstrated that radiation-inactivated tumour cells do not kill bystanding tumour cells. In most experiments, they even increased the colony forming efficiency of unirradiated cells or increased their take rate after transplantation. Also experiments in vitro that compared the radiosensitivity of cells in non-confluent monolayers with those in spheroids did not indicate any bystander effect (19). On the contrary, in those cell lines which display intercellular communication through gap junctions, radioprotection was observed (20). Something must be special about this study (18), the only one to show what might be called a bystander effect. The difference is related to the fact that the radiation source is present for a long time, first in the cluster and then in the petri dish, in which colonies develop. Although the likelihood that lethal activities of tritiated thymidine are released from killed cells and re-utilized by the bystander cells may appear low, more rigorous tests need to be performed to exclude this possibility before we can accept these experimental data as proof for the potential therapeutic effectiveness of the bystander effect in therapeutic nuclear medicine.

ROLE OF RADIATION-INDUCED GENOMIC INSTABILITY IN TUMOUR CURE BY RADIOTHERAPY

Whereas the potential role of the bystander effect is based on a somewhat controversial experiment, the role of radiation-induced genomic instability has been well documented and is in line with several experimental facts observed by different groups of radiobiologists. Suit was the first to demonstrate that the radiosensitivity of a recurrent tumour might be greater than the radiosensitivity of the original tumour (21). This is in contrast to the general perception, by which it is assumed that it is predominantly the inherently radioresistant tumour cells which survive at the end of radiotherapy and grow into the recurrent tumour. The same finding was reported later by Ando (22) and confirmed in unpublished experiments by Kummermehr, all in different experimental mouse tumour systems. The experimental procedure was always the same: The primary tumours were given curative radiation doses (usually a TCD90) and a recurrent tumour was selected for further experimentation, dissected and transplanted into fresh animals of the same isogeneic strain and, again, a tumour cure experiment was performed. In all three experiments, an increase in radiosensitivity was demonstrable as a significant decrease of the TCD-50 by 25–30%.

Suit (21) related this increased radiosensitivity to the phenomenon of small colony formation (23). Sinclair (23) reported that after high radiation doses many colonies arising from surviving Chinese hamster cells were growing continuously at a slower rate than before irradiation. These cells must have sustained a form of heritable radiation damage. Individual small and large colonies were isolated, expanded and investigated for growth rate, chromosome aberrations, plating efficiency and radiosensitivity. The growth rate of small colonies was persistently reduced two- to threefold (by a doubling of the generation time). Numerical and structural chromosome aberrations were equally common in the progeny of small and large surviving colonies. The most dramatic difference between large surviving clones and small surviving clones was a significant increase in radiosensitivity by 25%. Also the colony forming efficiency was reduced to about 50% of the normal value. Both features persisted in the clonal progeny for over a year in some clones, although some others reverted to the normal phenotype after a few months. Sinclair drew particular attention to the fact that poor plating efficiency and increased radiosensitivity were not specific for cells that survived irradiation, but applied to all clones that formed small colonies whether they were the progeny of irradiated or of unirradiated cells. In contrast, the radiosensitivity of cells derived from large clones was unchanged while the plating efficiency was variable but also reduced compared to control clones.

It is surprising that only a few experiments have been performed investigating these long-term changes in other irradiated cell lines. Even after radiobiologists focused on the phenomenon of radiation-induced genomic instability, its relationship to the small colony phenotype of surviving irradiated cells received little attention. Brown and Trott (24) showed that all 25 clonal progeny from 25 large surviving colonies of irradiated HeLa cells showed a significantly reduced colony forming efficiency by > 50% but

normal radiosensitivity. Also in Chinese hamster cells, the clonal progeny of large colonies surviving after X-irradiation (13) and alpha-irradiation (14) showed decreased colony-forming efficiency which was associated with an increased rate of chromosomal damage. There was no difference with regard to the reduction of colony-forming efficiency, or the frequency of micronuclei, or the frequency of unstable chromosome aberrations between the clonal progeny of small colonies derived from unirradiated cultures, and small colonies from irradiated cultures, and large colonies reverted from the progeny of small colonies, and large colonies derived directly after irradiation (25).

The experiments on the persistence of non-lethal radiation injury in the clonal progeny of surviving irradiated cells can be summarized as follows: In all experiments there was a significant reduction in colony forming efficiency, which was associated with a persistent increase in various unstable chromosome aberrations and other cytogenetic damage. A significant increase in intrinsic radiosensitivity was only observed in the progeny of cells that formed small colonies (23) and not in the clonal progeny of cells that form large colonies (23, 24), whereas the decrease in colony forming efficiency was similar in both. The increase in intrinsic radiosensitivity, however, was not directly related and specific to radiation damage, but occurred equally in the progeny of cells which formed small colonies, spontaneously.

Since Suit (21) and Ando (22) focused their studies on the radiosensitivity of local recurrences which grew very slowly and became apparent only >200 days after very high radiation doses, their interpretation that they represented the in vivo equivalent of the small colony forming survivor phenotype appears convincing. Since the recurrence arose after a dose that had cured most other irradiated tumours, it can be regarded as a monoclonal tumour, which would mean that only a cell that is 25% more radiosensitive than the bulk of the tumour cells survived radiotherapy. This is highly unlikely. Therefore it has to be postulated that this increased radiosensitivity arose as a consequence of a stable mutation in the first few cell divisions during clonal expansion. The frequency of small colony formation, however, is much too high to be consistent with a typical single gene mutation. It resembles much more the characteristic features of radiation-induced genomic instability.

The results of the determination of intrinsic radiosensitivity of the late recurrence by Ando (22) are consistent with the interpretation of a small colony phenotype; however, the variability of the data does not exclude other interpretations. For this reason, Tarnawski, in Kummermehr's laboratory, investigated this problem again (26) using the in vitro tumour megacolony system, which permits much more rigorous quantitative testing of the interpretations. The megacolony system is well suited for investigating the response of recurrent tumours, since it allows the simultaneous analysis of growth curves, the determination of megacolony radiosensitivity, i.e. cure and cellular radiosensitivity and, this way, the number of functional tumour clonogens in the megacolony over several culture passages.

The megacolony system, although it is an in vitro system, permits the investigation of tumour cells in a three-dimensional system, with close epithelial contact, marked heterogeneity of cell density and of cell proliferation similar to the conditions in tumour spheroids. We studied the radiosensitivity of recurrent megacolonies in two different experimental protocols (26): In the first experiment, the clonogen density in a large number of clones regrowing after a non-curative radiation dose was determined by irradiating them at different sizes and determining the rate of cure and recurrence as a function of size. In the second experiment, one megacolony recurrent after the TCD-50 was isolated, expanded and retreated. The growth rate of the recurrent megacolony was decreased by a factor of 3 compared to the original megacolony. The TCD-50 of the megacolony decreased from 24 Gy to 16 Gy, by 33%. Both findings are surprisingly similar to those in recurrent tumours in vivo (21, 22).

In both experiments, the radiosensitivity of the recurrent clones was significantly increased. The clone re-irradiation experiments were designed to estimate not only the overall clone radiosensitivity but also the two components that determine it, i.e. cellular radiosensitivity and the number of clonogens in the re-irradiated clones. According to this analysis, the cellular radiosensitivity did not change during the first 6 months after isolation of the recurrent clones. Therefore, we interpreted the increased radiosensitivity of the recurrent clones as the result of a decreased concentration of clonogenic cells in recurrent clones compared to pre-irradiation clones.

We ascribed the increased radiosensitivity of the recurrent tumour clones to the phenomenon of radiation-induced genomic instability, and in particular to its manifestation of delayed reproductive death (9). Furthermore, we interpreted these data within the framework of the tumour stem cell model (26) and concluded that the probability of self-maintenance of tumour stem cells, which is normally 78%, decreased in the recurrent clones to 55% in the same way and probably by the same mechanism that causes the persistent reduction of the colony forming ability in surviving cells due to radiation induced genomic instability. At each cell division of the surviving irradiated tumour cells the rate of de novo appearance of non-clonogenic cells was increased. This must lead to a decreased growth rate of the recurrence and a decreased proportion of clonogens in the recurrent tumour, thus making it more radiosensitive (1, 26).

CONCLUSION

There is no reason to doubt the validity of the old paradigm of curative radiotherapy, which states that local tumour control by radiotherapy requires the complete sterilization of all tumour 'stem' cells in the tumour volume. Neither bystander effects nor radiation-induced genomic instability would be able to contribute substantially to the probability of local tumour control of the primary cancer by radiotherapy. All proven experimental facts are consistent with the interpretation that a local recurrence after curative radiotherapy arises from a single or a few tumour 'stem' cells which, by chance, have not been lethally hit by the given radiation doses and retained their unlimited proliferative potential. However, the progeny of these surviving tumour 'stem' cells are likely to suffer from radiation-induced genomic instability, which results in the persistent appearance of non-stem cells, i.e. a reduced probability of self-maintenance. This leads to a slower growth rate of the recurrent tumour, a reduced stem cell fraction and, as a consequence, an increased radiosensitivity of the recurrent tumour. In some recurrent tumours, particularly those that develop very late and grow very slowly, radiosensitivity of the recurrent tumour may be further increased by increased intrinsic radiosensitivity which could be related to the as yet poorly defined phenotype of 'small colony formation' (23). There may be other consequences of radiation-induced genomic instability such as changes in chemosensitivity, but at present these are not facts, not interpretations, but speculations.

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