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THE USE OF DNA DOUBLE-STRAND BREAK QUANTIFICATION IN RADIOTHERAPY

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DNA double-strand breaks (DSB) are an important direct consequence of treating cells with ionizing radiation. A variety of evidence points toward DSBs being the key damage type linked to radiation-induced lethality. In particular, the link between DSB and chromosome breakage, which in turn closely correlates with cell death in some cell types, is strongly supportive of this concept. There has been much interest in the possibility of using measures of strand breaks as a pretreatment test of radiation response. This has largely been in the context of assessing inherent cellular sensitivity through damage induction or repair parameters. A number of studies have produced hopeful results, but overall there has been no parameter that can reliably predict radiosensitivity. This may be due to the inadequacies of the assays, but it is more likely to reflect the fact that the radiosensitivity of cells is dictated by a whole series of events; alterations in many of these can alter the overall response. In addition, it is now recognized that cell-signalling pathways form an essential part of the cellular response to damage, and these can be triggered by damage other than DSB. It is therefore possible that while DSBs are clearly important—and they may be the single most important lesion in some types—other damage types may be significant triggers of cell death pathways after ionizing radiation treatment.

Radiation, Double-strand break, Repair, Damage.

INTRODUCTION

Ionizing radiation induces a vast number of damage types in DNA. Small-base or nucleotide damage lie at one extreme with single- and double-strand breaks, and multiply damaged sites at the other. DNA double-strand breaks (DSBs) have been considered to be the most important type of lesion for the cytotoxic effects of radiation because of findings suggesting that their levels vary in directions consistent with killing. In addition, artificially induced DSBs produce similar cellular effects to radiation. In particular, patterns of chromosomal damage are similar in irradiated and restriction enzyme–treated cells (1). The close correlation between chromosome fragment production and killing in many cell systems has been important in linking DSB to death, because it is a natural step to relate DNA strand breakage to chromosome breakage. It has therefore been logical to measure DNA DSB induction and repair in human cells and relate the results to clinical effectiveness of radiotherapy with a view to explaining the outcomes, predicting response, and designing treatments that may change the clinical results. In this short review, we aim to consider the significance of the measurement of DSB in some of these approaches.

Assays of DNA double-strand breaks

All of the major methods for measuring DSB frequency essentially measure the size of DNA strands. Some of the first assays measured the rate of sedimentation of DNA through sucrose, which is dependent on DNA fragment size. These methods had a strong physical basis but had the disadvantage that they were not sensitive to biologically relevant doses. The next phase saw the extensive use of filter elution methods that quantified passage of DNA through pores in filters. These were sensitive at lower doses than sedimentation but were still not ideal. In the main they were not suitable for clinical material, because most methods required radioactive labeling of the DNA and large numbers of cells.

Major steps forward were made with the introduction of electrophoretic methods. The single-cell gel electrophoresis

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(SCGE, "comet") assay requires few cells so is ideal for clinical samples, but the neutral version of the assay that is needed for DSB quantification still lacks sensitivity in most laboratories. Pulsed-field gel electrophoresis (PFGE) is now the method of choice for many people. It is usually sensitive down to a few Gy, has a modest requirement in terms of cell number, and while most laboratories use radioactive labeling on cell cultures, it can be used on unlabelled cells. A further advantage of PFGE is that it can be modified to incorporate the assessment of fragmentation of small DNA regions so that variation in damage within the genome can be assessed. In addition, there are reports of repair fidelity being assessed with PFGE-based methods, and if these can be developed further, they should provide important insights into DNA damage induction and processing (2).

Thus there are now methods available to quantify DSB at clinically relevant doses and on clinically obtainable cell numbers. It is important to consider, however, some of the potential problems with these methods. One of the key factors is background damage in unirradiated human cells. This can be high for human cell lines and is often even higher in cells direct from the patient. This can severely limit the sensitivity of the experiments and may well influence the results obtained due to the nonlinearity of the dose–response curves in most cases. In the case of PFGE, it is usual for the response to saturate when around 60% of the DNA has migrated from the well in the gel. If the background level becomes a significant proportion of this 60%, erroneous curves will result.

Heterogeneity of the cell population is also a potential problem. This has been examined in detail using the SCGE assay that examines each individual cell. It is clear that cells migrate in electrophoretic gels differently depending on the phase in the cell cycle in which they reside, probably due to differences in chromatin structure (3). Thus, if the variation in cell cycle distribution is substantial, the assay can produce spurious results. If samples are measured that were irradiated *in vivo*, spatial heterogeneity may be reflected in the results obtained. This has been used to advantage with the SCGE assay, because Olive *et al.* have gained measures of cell cycle parameters and hypoxic cell fractions by enumerating the number of cells in populations that are resistant to migration in the gel (4). Of course separating these parameters from genuine variation in the sensitivity to damage induction and repair is one of the key factors to be considered in this field.

Heterogeneity in the type of DNA damage may also be an important issue. It has been suggested that the reparability of DSB varies depending on what other lesions are induced in the immediate vicinity (i.e., other components of multiply damaged sites). There is also the potential for nonrandom distribution of damage in the genome because of the recognized protective effects of histones attached to the DNA (see below); while there are only small amounts of evidence for repair of DSB being influenced by transcription activity, it must be likely that the binding of repair complexes is influenced by the presence of other DNA-associated proteins. This heterogeneity of damage is also important in a practical sense, because not every method of detection may be sensitive to the same lesions. Woudstra *et al.* (5) have examined the damage levels detected in two cell lines by a range of methods, and the results suggest that different assays can lead to different conclusions. The key question here is which is the most relevant to the cell; with current understanding, this is impossible to answer. It is logical to use conditions that seem as close as possible to the cellular environment, but that assumes the cellular damage recognition system works in the same way as the detection systems used experimentally; this clearly need not be the case. Olive (6) has recently suggested that even identical lesions may be recognized differently by different cells, which may add a further level of complexity onto any attempts to come up with an all-encompassing DSB end point that correlates with radiosensitivity.

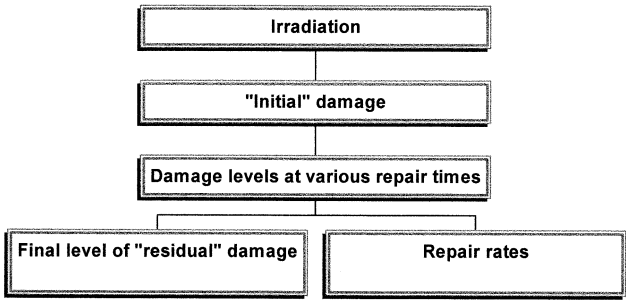

Fig. 1. Stages in the damage induction and repair sequence that are measured to provide information of potential relevance to radiotherapy.

Which parameters are measured?

Figure 1 outlines the stages in the post-irradiation processing in which DNA damage measurements are commonly made. While the physical deposition of energy should be similar for all cells, there are soluble and chromatin-associated factors in cells that can influence the amount of damage inflicted in DNA (7). This is demonstrated in Table 1, where the amount of damage inflicted is shown to be highly dependent on the presence of soluble and DNA-associated molecules (8). Once a damaged DNA molecule exists in a cell, a variety of repair processes become active. The enormous strides forward in our understanding of repair of DNA DSBs in recent years have placed an emphasis on the process of nonhomologous end rejoining as the primary DSB repair process in mammalian cells (9). However, it is now evident that there is a role in mammalian cells for homologous recombination repair in rectifying DSB (10). To quantify DSB—the initial damage, the rate of rejoining, and the final level of damage remaining after repair appears to be complete— the primary end points have been evaluated, as discussed below.

Initial damage

Initial damage has a practical definition in the context of these studies. It is the amount of damage detected when

Table 1. DNA damage in different chromatin substrates from RT112 cell line

Condition	Treatment	Chromatin state	DNA retained after 30 Gy
A	Whole cells	All soluble and chromatin-associated scavengers	64%
B	Lysed cells	A - soluble scavengers	52%
C	0.35 M NaCl	B - non-histone proteins	47%
D	0.6 M NaCl	C - histone H1	24%
E	1.2 M NaCl	D - histones H2A/B	10%
F	2.0 M NaCl	E - histone H3 and H4	9%
G	Proteinase	Naked DNA	6%

Damage is expressed as the percentage DNA retained in a pulsed field gel electrophoresis gel as described in Mateos *et al*. (8). Data adapted from Mateos *et al*. (7).

experimental temperatures are maintained at 4°C. Thus it is likely to reflect the position at the end of the energy deposition events and the rapid chemical processes that occur in an irradiated cell. With PFGE the dose range can be realistically kept below 30 Gy, and sensitivity can be achieved at 2–5 Gy. Figure 2 shows the initial damage detected in two human cervix carcinoma cell lines. The data demonstrate the range of doses used and the common variation between cell lines in the slope of the damage induction curve. This variation in damage induction is demonstrated in Fig. 3, where the slope of the damage induction curve is related to sensitivity to the killing effects of radiation represented by the surviving fraction at 2 Gy (SF2). In this combination of data from several studies, it can be seen that there is a relationship between these two parameters. Although it is very common for the examination of this end point to produce variation between cell lines, it has been a matter of considerable debate as to whether this has true biologic relevance. As stated above, this debate is not possible to resolve at present due to our lack of understanding of damage recognition, but the correlation shown in Fig. 3 may have practical implications in that there are few DNA damage end points that have been shown to parallel radiosensitivity over such a wide range of tumor cell lines (See below).

Repair rates and residual damage

Studies with human cells demonstrate that DSB rejoining occurs primarily over a 2-h period, although there is some evidence of further rejoining up to 24 h after treatment (11).

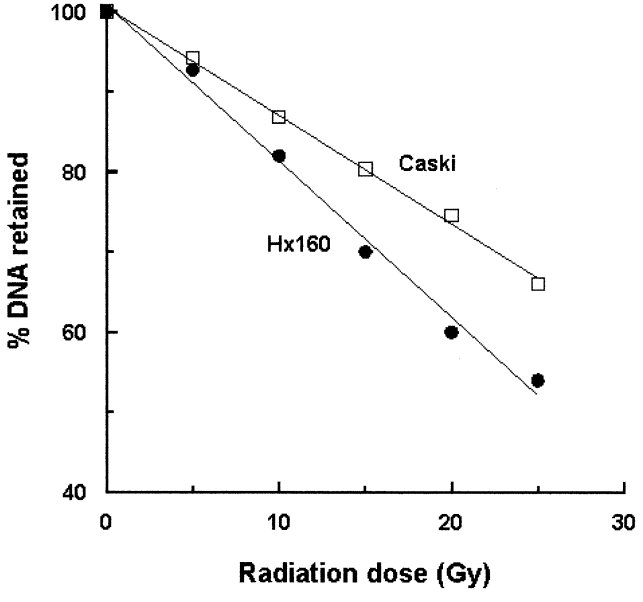

Fig. 2. Pulsed-field gel electrophoresis analysis of damage induction in two human cervix carcinoma cell lines. Damage is expressed as DNA retained in a PFGE gel after single doses of radiation. Methods are described in Ref. 11, and points are means of three independent experiments.

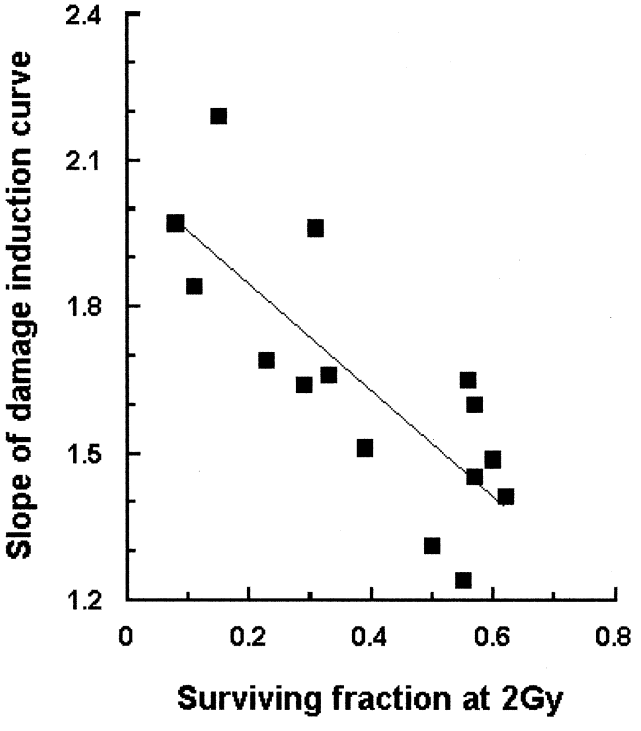

Fig. 3. Relationship between damage induction and radiosensitivity. Damage induction is given as the slope of damage induction curves as described in Fig. 2. Radiosensitivity is given as surviving fraction at 2 Gy from clonogenic assays. Data are from a range of human tumor cell lines with a combination of previously unpublished data on 7 cervix carcinoma cell lines and 8 other human tumor cell lines described in Ref. 11.

The decrease in DSB has been described as a simple exponential in some studies and biphasic in others. Repair rates can be derived from such analyses, and these usually produce a half-time of around 10–20 min for a rapid phase and 1–2 h for the slow phase where biphasic kinetics are used (12). In some repair-deficient mutants, the deficiency in DSB rejoining is so marked (e.g., xrs) (13) that the precise mathematical approach is not of immediate importance. However, if a mechanistic interpretation is intended, different approaches can be significant. For example, a biphasic nature is usually explained in terms of the presence of two different types, but a recent analysis by Foray *et al.* (14) suggests that most rejoining kinetics may be consistent with the existence of a wide range of lesion types, each with its own level of reparability. In the study of human tumor cells, such subtleties are rarely significant, because the differences found have generally been very small, and the precision of the data is commonly insufficient to allow in-depth analysis. Nevertheless, some correlations have been detected between radiosensitivity and DSB rejoining rates, which at least support the belief that repair is an important determinant of radiosensitivity in human tumor cells (12).

It has been generally found to be stretching DNA damage assays to their limit to examine residual damage several hours after treatment unless extremely marked effects are evident, such as with xrs cells. Human tumor cells are subject to problems of high background damage and proliferation during extended experiments, which makes them difficult to examine. The most success has been had with the examination of residual damage in human fibroblasts where the maintenance of a nonproliferative state greatly assists the experiments (15). Using this approach, increased residual damage that was not evident with earlier DSB assay methods has been detected in ataxia telangiectasia cells (16).

Are DSBs the whole story?

As mentioned above, the DSB has received the most attention among the various types of damage inflicted by radiation, primarily because of the close relationship to chromosome damage, which in turn is closely linked to cell death in many cell types. However, the recognition that apoptosis may be an important mode of radiation-induced death in some cell types raises the possibility that other types of damage may be important triggers for the appropriate cell signaling pathways. It is recognized that DNA-damaging agents that do not produce DSB can induce apoptosis, so the determination of the primary mode of cell death may be an important factor in evaluating the usefulness of DSB measurements. Even if DSB is the important lesion for cell signaling, the question needs to be asked: At what point after irradiation is the damage detected, and therefore at what point in the damage induction and repair sequence is the level of damage critical? If DNA damage is detected rapidly, the efficacy of repair might have a reduced importance, because adverse consequences may be triggered before the repair process having a significant beneficial effect. Also the study of synchronized cells might produce erroneous results, because the time between irradiation and the cell hitting a particular point in the cell cycle where damage is detected might be an important variable in proliferating cells. These are questions raised by the recent advances in our knowledge of the cellular response to DNA damage, and they now need to be integrated into DSB analysis procedures.

Is there a role for quantification of DSBs in radiotherapy?

There are two main areas in which quantification of DSBs might have a place in radiotherapy. In a predictive testing setting, any assay that can give a rapid and accurate indication of the sensitivity of the cells within a tumor and/or the cells in the normal tissues will potentially have a large benefit for radiotherapy planning. To date data on human cell lines have produced a range of possible end points of damage induction and repair that may be useful (17); there have been some studies that have examined these on biopsy material. In most cases the precision, reproducibility, and relationship to radiosensitivity are still not adequate for clinical application. The reasons for this are probably largely technical, but the questions posed in the previous section may contribute to this. It is likely, therefore, that knowledge of the physical breaking of the DNA will need to be combined with a broader view of the biologic response to damage. To turn this around, it is also important to recognize that DNA damage is at the heart of the effects of radiation; thus the use of other biologic end points to predict radiosensitivity needs the knowledge of DNA damage levels to enable full interpretation.

With the continuing development of gene therapy technology, there comes the opportunity for sensitizing tumor cells to radiation by targeting genes involved in DSB processing. It is clear that in the development stages, there is a need for DSB measurements to confirm the effect of these new strategies; however, in the long term it needs to be remembered that the limiting step that determines radiosensitivity may well differ in different tumors. Thus the concept of predicting susceptibility to genetic sensitization needs to be adopted for these approaches to be targeted at those who will benefit most.

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