Stable Translocations Detected by Fluorescence \textit{in Situ} Hybridization: A Rapid Surrogate End Point to Evaluate the Efficacy of a Potentiator of Tumor Response to Radiotherapy\textsuperscript{1}

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\textbf{ABSTRACT}

Testing potential modifiers of the response of tumors to radiation therapy requires large, expensive, and time-consuming clinical trials. It would, therefore, be of value to have a rapid surrogate end point of tumor response that could be used to evaluate such modifiers. We here propose that radiation-induced stable chromosome translocations measured by fluorescence \textit{in situ} hybridization (FISH) could fulfill this purpose. The assay requires that the ratio of nonlethal stable translocations to lethal dicentric aberrations be unity and not change with radiation dose and that radiation-induced stable translocations remain in the tumor cell population essentially indefinitely after irradiation. We have tested these assumptions with four human tumor cell lines \textit{in vitro} at doses of 1–5 Gy and found them to be valid. We also modified the response to fractionated irradiation of a human tumor xenograft in three different ways and quantitated the tumor response using clonogenic cell survival and using the FISH stable translocation assay. Both assays gave similar values for the extent of radiation modification. These data suggest that this assay could allow clinical evaluation of potential radiation sensitizers with fewer patients and in shorter times than is the case with conventional clinical trials.

\textbf{INTRODUCTION}

A considerable number of modifiers of the radiation response of tumors have been identified in preclinical studies and are awaiting clinical investigation. These include the hypoxic cytotoxin TPZ\textsuperscript{4} (1), carbone with nicotinamide (2), recombinant human erythropoietin (3), fluorocarbon emulsions, such as perfluorobutane (4), halogenated pyrimidine analogues (5), and others. Unfortunately, even with promising preclinical data, it will be extremely difficult to test all of these modifiers of tumor response using clinical outcome as the end point because of the large number of patients needed and the time and expense involved. Even for any one agent, there are questions that can only be answered by clinical testing, such as the optimum frequency, timing, and dose of the agent. It would, therefore, be of great practical value in radiotherapy if an inexpensive and rapid surrogate end point for tumor response could be used that would reduce the time, expense, and number of patients needed to be tested with any agent to determine its efficacy.

Based on the close relationship between radiation-induced chromosome aberrations and cell killing (6–8), we have proposed that the radiosensitivity of tumor cells in individual human tumors could be assessed \textit{in situ} using an assay based on chromosome aberrations scored in a single (or two to three) chromosome(s) using FISH with whole chromosome probes (9). However, although there is a close correlation between radiosensitivity and radiation-induced chromosome aberrations for most cell lines, there appear to be exceptions (10). This could cause complications if treatments for individual patients are to be tailored based on the incidence of radiation-induced aberrations in the tumor cells.

We here propose a new use of measurement of radiosensitivity using chromosome aberrations detected by FISH that would not be affected by individual exceptions to the relationship between chromosome aberrations and cell killing. We suggest that this technique can be used to measure modification of radiosensitivity of tumors \textit{in situ}. In particular, we believe that chromosome aberrations assessed by FISH can be used to assess the efficacy of a potentiator of radiation damage to tumors. The assay we propose would measure stable translocations in biopsy (or fine needle aspirate) specimens taken from tumors that have been treated with or without the modifying agent. If properly validated, this assay could have significant practical value by allowing the assessment of new modifying agents (such as radiation sensitizers) without having to perform clinical trials using local control or survival as the end point with every agent. The goal of such a surrogate end point would be to allow clinical testing of many more agents and/or combinations so that the traditional clinical trials could be reserved for the most promising agents.

It has been known for many years that exchanges between chromosomes following irradiation damage can be either lethal dicentrics with acentric fragments or nonlethal stable translocations. We have shown previously using FISH, as have others, that stable translocations are induced in normal cells in culture with equal probability to dicentric exchanges and remain in the population unchanged with multiple divisions, whereas dicentrics are lost from the population due to cell death (11–13). The basis of our proposal that stable translocations can be used to assess potentiation of radiation damage is that such potentiation would be equivalent to increasing the radiation dose, which would be expected to increase both dicentric aberrations and stable translocations to the same extent. Because the incidence of stable translocations can be readily measured, then, given several assumptions, the increased yield of dicentrics and, therefore, the expected increase of cell killing can be estimated.

The purpose of the present study was to examine in four different human tumor cell lines \textit{in vitro} the two principal assumptions inherent in the use of stable translocations to assess tumor modification and then to test the assay in a human tumor xenograft. The two assumptions we have examined are the numerical equality of radiation-induced dicentrics and stable translocations in these tumor cell lines and the assumption that stable translocations remain at an undiminished level after several divisions following irradiation. As discussed above, these assumptions have been tested and validated by a number of investigators but only for normal cells (lymphocytes and fibroblasts). However, both assumptions have been questioned recently for tumor cells (14). The data we present for these four human cell lines \textit{in vitro} and one xenograft \textit{in vivo} are consistent with the validity of the assumptions inherent in the assay. In particular, we show that for these four human tumor cell lines, the ratio of dicentrics to translocations is indistinguishable from unity at doses of 1–5 Gy and that...
stable translocations remain in the population at an undiminished level for many postirradiation divisions. In addition, our data show that for three different modifiers of response of a human tumor xenograft to fractionated radiation, the extent of modification determined by clonogenic assay is indistinguishable from that determined using the FISH stable translocation assay. We, therefore, believe that this assay has considerable promise for use to assess the efficacy of any modifier of radiation damage in the clinic.

MATERIALS AND METHODS

Mice and Tumor Cell Lines. The mice used in this investigation were immunodeficient SCID mice, which were bred and housed under specific pathogen-free conditions in the American Association for Accreditation of Laboratory Animal Care approved facility at Stanford Medical Center. The HT1080 and A549 cells were obtained from the American Type Culture Collection, Rockville, MD, and were maintained in α-MEM + 10% fetal bovine serum. The FaDu tumor cell line, originally derived from a human hypopharyngeal carcinoma, was obtained from Dr. Herman D. Suit (Massachusetts General Hospital, Boston, MA), and the SCC61 tumor cell line, originally derived from a head and neck carcinoma, was obtained from Dr. Jeffrey Schwartz at the Argonne National Laboratory.

Tumor Irradiation Technique. A549 human tumor xenografts were obtained by implanting 5 × 10^2 cells intradermally on the dorsum, near the base of the tail, and were irradiated locally using a 250 kVp X-ray machine. Unanesthetized SCID mice were placed in lead boxes through which the tumors protruded for irradiation. The irradiation procedures and characteristics of the lead boxes have been published previously (15). Briefly, however, the dose rate was 1.69 Gy/min with added filtration of 0.35-mm copper, 15 mA, target-to-tumor distance of 31 cm, and a half-value layer of 1.3-mm copper. In experiments involving breathing of gases other than air, the jig holding the lead boxes was placed inside a lucite container through which the gas was passed.

TPZ, Carbogen, and 10% Oxygen Breathing. TPZ (SR 4233; 3-amino-1,2,4-benzotriazine 1,4-di-N-oxide; from Sanofi-Winthrop, Great Valley, PA) was dissolved in sterile saline at a concentration of 1 mg/ml and delivered i.p. at 0.12 mmol/kg 30 min before each irradiation treatment. Nicotinamide (Sigma Chemical Co., St. Louis, MO) was dissolved in sterile saline at 25 mg/ml and delivered i.p. at 1000 mg/kg 1 h before each irradiation treatment, with carbogen given 5 min before and during irradiation. This timing of 5 min before and during irradiation was also used with 10% oxygen breathing. We have shown in preliminary experiments that this 5-min preirradiation breathing period is optimal, because there was no increased or decreased radiosensitivity with carbogen or 10% oxygen breathing for longer periods.

In Vivo-in Vitro Excision Assay. The excision assay to measure clonogenic survival in vitro was used to assess the response of the A549 tumors to fractionated irradiation (6 × 2 Gy, two fractions daily). Briefly, however, at approximately 18 h after the final irradiation dose to the A549 tumors, the tumor-bearing SCID mice were killed, and separate single cell suspensions were prepared from each tumor by enzymatic digestion (16). Each suspension was then split in two, with one-half being plated for passage in vitro for detection of chromosome aberrations and the other half counted and plated into 60- or 100-mm polystyrene Petri dishes at appropriate dilutions to give approximately 50 colonies/dish. Plating efficiencies for the untreated tumors were approximately 30%. Surviving fractions were calculated on the basis of total clonogenic cells per tumor (surviving fraction × cell yield/tumor).

Detection and Scoring of Stable Translocations with FISH. FISH was performed on the metaphase spreads using whole chromosome probes in combination with pancentromeric probes. Probes for human chromosome 1 or chromosome 4 were used to score translocations in the four human tumor cell lines. These chromosomes were chosen because they were diploid and intact in these cell lines.

The technique for preparation of the probes and in situ hybridization is a modification of the procedure described by Pinkel et al. [Ref. 17; as detailed by us (18)]. Briefly, the chromosome probes were prepared by nick translation with biotin-dATP (Bethesda Research Laboratories, Gaithersburg, MD), and hybridization was performed under a coverslip on the denatured chromosomal DNA. Different amounts of unlabeled genomic DNA, based on the probe concentration and the fraction of the genome represented by the probe, were added to the hybridization mix to deplete the labeled copies of shared sequences, thereby increasing the contrast. The hybridization mix was incubated for 5 min at 70°C and prehybridized for 1–2 h at 37°C before being applied to slides under a glass coverslip. After hybridization for 3–5 days at 37°C, the slides were washed thoroughly and stained by applying alternating layers of avidin-fluorescein and biotinylated goat anti-avidin. Between the avidin and goat anti-avidin treatments, the slides were washed 2 min in three changes of PB buffer (0.1 M sodium phosphate dibasic). A fluorescein antifade solution containing propidium iodide was then added to counterstain the rest of the chromosomes red. The slides were viewed and scored “blind” (i.e., without knowledge of the treatment) with a Nikon Optiphot fluorescence microscope equipped with a VFXII camera. Translocations (and dicentrics) were scored as bicolor (red/yellow) chromosomes and classified as translocations or dicentrics based on the pancentromeric probe (Fig. 1).

Pancentromeric probes to all human centromeres were generated by PCR using primers designed by Weiter et al. (19) to specifically amplify the 171-bp α satellite repeat from human genomic DNA. After 35 cycles, the ∼171- and ∼350-bp products were gel purified and subsequently used as template for an additional 20 cycles in the presence of 50% biotin-14-dATP (Life Technologies, Inc., Gaithersburg, MD) to label the centromeric probe.

The hybridization protocol for the simultaneous visualization of both the pancentromeric probe and whole chromosome probe consisted of the sequential addition of the probes to the denatured slide. Pancentromeric probe was denatured and applied to slides for 1–2 h, and then the coverslip was removed and the whole chromosome probe was added (as described above) for 3–5 days. Because both probes were biotinylated, they could be stained directly with avidin fluorescein.

Statistical Analyses. All error bars on the data for the incidence of aberrations were calculated assuming a Poisson distribution of dicentrics or stable translocations. The data for stable translocations as a function of time were fitted with least squares regression lines with each point weighted inversely by the square of the error on that point. Because none of the cell lines showed a significant trend of translocations with time, the data were then fitted with a weighted least squares regression line with a zero slope. The data for translocations and dicentrics as a function of dose were fitted with linear-quadratic dose-response curves with each point again weighted by the inverse of the square of its error. Because none of the four cell lines showed a significant difference between the parameters of the curves for dicentrics and translocations, the exchange data were pooled and refitted with the best common curve. All curve fitting and statistical analyses were performed using Sigma Plot 5.0 for Macintosh.

RESULTS

Frequency of Stable Translocations as a Function of Time after Irradiation. Fig. 2 shows the data obtained for the four human tumor cell lines irradiated under exponentially growing conditions with 5 Gy (and 3 Gy for the SCC61 cells) and assayed for translocations and dicentrics as a function of time up to 360–500 h (15–21 days) after irradiation. All of the metaphase spreads were hybridized jointly with a pancentromeric probe and a single chromosome probe (chromosome 4 for A549, FaDu, and HT1080; chromosome 1 for SCC61). The use of the pancentromeric probe usually provided an unambiguous method of distinguishing between dicentrics and stable translocations (Fig. 1).

It can be seen from Fig. 2 that the incidence of dicentrics falls rapidly with time after irradiation, reaching a level indistinguishable from zero by 100–200 h after irradiation. It is likely that the values of dicentrics above zero after 100–200 h (for example, in the HT1080 and SCC61 cells) represent occasional translocations mis-scored as dicentrics. In marked contrast to the loss of dicentrics, there is no significant trend for a decrease (or increase) in translocations with time after irradiation for any of the four cell lines.

Dose-Response Curves for Dicentrics and Translocations. To test the assumption that the ratio of translocations to dicentrics is equal to unity at the first mitosis after irradiation and is independent of radiation dose, we obtained a dose-response curve for both classes.
of aberrations using the combination of whole chromosome FISH probe and pancentromeric probe. The data for the four cell lines are shown in Fig. 3. We fitted each set of data (translocations and dicentrics) to a weighted least squares linear-quadratic dose-response curve. Because we found no significant differences between the linear and quadratic parameters for the individual plots for translocations and dicentrics for any of the four cell lines, the lines shown are the best fit to the pooled data. As a further test of the equality of translocations and dicentrics, we fitted least squares regression lines through the ratios of translocations to dicentrics as a function of dose for each of the cell lines. For each line, we found that the ratio of translocations to dicentrics was not significantly different from unity and showed no trend as a function of dose. We conclude from these data, therefore, that although there are apparently differences in the dose-response curves for the four cell lines, the incidence of translocations and dicentrics as a function of radiation dose are indistinguishable and can be fitted to a linear-quadratic dose-response curve.

Analysis of Modification of the Response of a Human Tumor Xenograft to Fractionated Irradiation Using Clonogenic Cell Survival and Stable Translocations. As a further test of the hypothesis that stable translocations can be used to assess modification of the response of tumors to fractionated radiation, we performed an experiment in which SCID mice bearing the A549 transplanted human tumor xenograft were irradiated with six fractions of 2 Gy (two fractions daily) under normal air breathing conditions or with three different potential modifiers of the radiation response. The three modifiers were that the animals breathed 10% oxygen or breathed carbogen during irradiation or were given a dose of the hypoxic cytotoxin TPZ 30 min before irradiation. In the case of the carbogen-breathing group, the animals were also injected with nicotinamide (1000 mg/kg) 1 h before irradiation. It was expected that breathing 10% oxygen would increase tumor hypoxia, thereby reducing the response of the tumors to fractionated irradiation, whereas breathing carbogen (with nicotinamide) would increase tumor oxygenation and hence increase the radiation response. We have also shown previously that TPZ can significantly increase the response of murine tumors to fractionated irradiation (20). At approximately 18 h after the sixth radiation dose, the animals were sacrificed, the tumors were removed, cell suspensions were made, and the cell suspensions from each tumor were divided into two, with one-half being used to assess clonogenic survival and the other half plated in vitro and passaged for 3–4 weeks to allow for cell death and the elimination of dicentrics from the population. Metaphases were then collected and translocations scored using a whole chromosome 4 probe with a pancentromeric probe. The results are shown in Fig. 4. It is clear from this experiment that we were able to modify the response of the tumors by the three different treatments: breathing 10% oxygen protected the tumors, whereas both TPZ and carbogen with nicotinamide sensitized the tumors to fractionated radiation. Furthermore, despite the very different responses of the tumors to the four treatments, a common line could be fitted showing a similar relationship between clonogenic survival and translocations in chromosome 4 (Fig. 4, left panel). Thus, the dose modification factors assessed for the three different treatments were not significantly different when assessed either by clonogenic survival or by stable chromosome translocations (Fig. 4, right panel).

DISCUSSION

The purpose of the present investigation was 2-fold: (a) it was to test two major assumptions inherent in the use of stable translocations to assess the modification of tumor response to radiation. For this we used four human tumor cell lines in vitro; and (b) it was to test the ability of this assay to provide a quantitative assessment of the response to fractionated irradiation of a human tumor xenograft treated with different radiation-modifying procedures.

The use of FISH with specific whole chromosome probes to meas-
ure translocations in human tumor cells and to provide a quantitative assessment of the modification of radiation damage depends on a number of assumptions as follows:

(a) That chromosome damage to the whole genome can be assessed using one (or a small number of) individual chromosome(s). Inherent in this assumption is that chromosome aberrations occur randomly throughout the genome so that their incidence in some of the chromosomes can be scaled to the whole genome, irrespective of the chromosomes involved. The reason for the importance of this is that the development of FISH with chromosome-specific probes, which allows the selective visualization of single human chromosomes (17, 21), has allowed the simple and unambiguous detection of exchanges in metaphase chromosomes. Although there have been many reports in the literature analyzing translocation breakpoints in lymphocytes and fibroblasts following irradiation that have suggested that certain chromosomes are over- or underrepresented in the distribution of breakpoints, there has been no agreement as to which chromosomes are so involved (12). Furthermore, when FISH has been used to analyze radiation-induced translocation breakpoints, the data obtained are consistent with random breakpoints throughout the genome (12, 22). Although all of these studies have been performed with normal cells, either in vitro or in vivo, there is no reason to believe that the conclusions would be different for tumor cells. Consistent with this is that for the vast majority of tumor cells, their radiosensitivities are correlated with breaks and exchanges in a single chromosome (10, 14, 23–25). In the case of aneuploid tumors with other than two copies of the "painted" chromosome(s), normalization of the translocation frequency based on chromosome number would probably be necessary.

(b) That stable translocations remain in the population at an undiminished level following irradiation, irrespective of the number of cell divisions of the population. This assumption, which has been validated by a number of investigators, including ourselves, for normal cells (12, 22) has recently been questioned for some tumor cell lines (14). We, therefore, felt it important to investigate this phenomenon using human tumor cell lines irradiated in vitro, including one of the same cell lines (SCC61) for which it has been reported that "stable" translocations are lost from the population following radiation. Our data are shown in Fig. 2. These experiments were performed with labeling of the chromosomes with both a pancentromeric probe and a whole chromosome probe to allow unequivocal identification of di-
centrics and translocations. We found no evidence for loss of stable translocations with time after irradiation. There is no obvious reason for the discrepancy between our data and those of Coco-Martin et al. (14) other than the possibility that their classification of "stable" translocations also included dicentrics.

(c) That the ratio of translocations to dicentrics is unity and is independent of radiation dose. On theoretical grounds, one would expect the ratio of translocations:dicentrics to be 1:1 (26). Several authors, including ourselves, have shown this to be the case for normal cells (12, 27). Nonetheless, there has been controversy over the ratio, although much of this is probably due to inadequate recognition of the two types of exchanges (13, 27). Although the validity of our assay does not depend absolutely on the ratio of dicentrics to translocations being 1:1 in all tumors, a variable ratio from tumor to tumor would introduce random errors into the assay. Also, the assay depends on the ratio not changing with radiation dose. Our in vitro
data show that the ratio is not significantly different from unity in the four tumors studied and is independent of radiation dose from 1–5 Gy (Fig. 3).

As a further test of the hypothesis that stable translocations could be used to assay modification of damage to fractionated irradiation, we performed an experiment in which one human tumor (A549) was treated in vivo with radiation doses comparable to those used in radiotherapy (2 Gy) with or without one of three different modifiers of radiation damage. Then, the cells were grown in vitro for 3–4 weeks following their in vivo radiation. For each of the modifiers, the degree of modification (quantitated as the dose modification factor) obtained using clonogenic cell survival was not significantly different from that obtained using stable translocations.

Assuming that this assay can be validated with further experiments and radiation-induced translocations can be assayed by biopsies of human tumors undergoing radiotherapy, the assay that we propose will have a number of attractive features for use in clinical radiotherapy. These include the fact that it can be used to study a radiation modifier (usually a radiosensitizer) under clinical conditions of fractionated radiotherapy, that it can be performed much more quickly and with fewer patients than can a conventional clinical trial, and that it has the potential of being simple; not a great deal of training is necessary to enable technicians to prepare chromosome spreads and to score translocations using the FISH technology, particularly if commercial chromosome painting kits are used.

In summary, we have demonstrated that radiation-induced stable translocations in single chromosomes, which are easily scored using whole chromosome FISH probes, can be a simple method to assess the efficacy of any radiation potentiator in human tumors. This assay could allow clinical evaluation of new potential radiation sensitizers with fewer patients and in shorter times than is the case with conventional clinical trials.
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