Use of the Comet Assay to Identify Cells Sensitive to Tirapazamine in Multicell Spheroids and Tumors in Mice

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ABSTRACT

Tirapazamine, a bioreductive drug preferentially toxic to hypoxic cells, produces significant numbers of DNA single-strand breaks that can be detected using the alkaline comet assay. Our goal was to determine whether single-strand breaks measured using this assay could act as a surrogate end point for cell killing in multicell spheroids and solid tumors in mice. In spheroids composed of Chinese hamster V79 cells, WiDr human colon carcinoma cells, or SiHa human cervical carcinoma cells, histograms of tail moments (indicators of DNA damage in the comet assay) could be used to identify the percentage of cells that sustained sufficient DNA damage to cause cell death after treatment with tirapazamine. The proportion of comets with tail moments \( \leq 20 \) (i.e., with damage comparable to that produced by about 10 Gy) correlated with cell survival irrespective of cell type, dose of tirapazamine, time of treatment, or position of cell in the spheroid. Single-cell suspensions from squamous cell carcinoma VII tumors in C3H mice or SiHa xenografts in severe combined immunodeficient mice were also analyzed for clonogenicity and DNA damage. Again, the percentage of comets with tail moments \( \leq 20 \) was found to be a good predictor of cell killing for both tumor types, providing tumor samples were obtained no more than 1 h after i.p. drug administration. Because tirapazamine is currently undergoing clinical trials, application of this procedure could provide an early indicator of drug administration. Because the tumor cells that survive treatment with a DNA-damaging drug are likely to be those that do not harbor extensive DNA damage following treatment, a DNA damage-based assay is appealing as a surrogate for cell killing.

Tirapazamine (SR4233) is a bioreductive drug that is preferentially toxic to hypoxic mammalian cells (5, 6). Phase I and II clinical trials are currently underway to evaluate the use of this drug in combination with radiation and chemotherapy. We have previously used the alkaline comet assay to measure DNA damage in multicell spheroids, murine tumors, and normal tissues exposed to tirapazamine (7, 8). This drug produces large numbers of DNA single-strand breaks in hypoxic cells even at nontoxic drug concentrations. Both strand breaks and cell killing increase with dose and time of exposure, suggesting that strand breaks might correlate with (if not cause) cell killing. Within subpopulations of spheroids and tumors, cell-sorting experiments indicated that cells that sustained the most DNA damage were also the most likely to die following treatment (7). However, if DNA damage is to be used as a surrogate for cell killing, it is necessary to show that a specific amount of DNA damage is predictive for cell killing independent of the dose of the drug, hypoxic status, time of exposure, or cell type. Therefore, we tested whether DNA damage by tirapazamine, measured using the comet assay, could be an effective predictor of cell killing in multicell spheroids and tumors in mice and might therefore have potential applicability in the clinic.

INTRODUCTION

Many genetic, pharmacological, and microenvironmental factors can influence the response of a tumor to a specific cytotoxic therapy. Although it is important to understand how all of these factors individually affect treatment outcome, a more immediate and practical problem is to simply estimate the overall impact of all genetic and epigenetic factors on tumor cell killing by a specific cytotoxic treatment. In an effort to develop a rapid assay to assess tumor susceptibility to treatment, we have examined the ability of the comet assay to act as a surrogate end point for cell killing.

In 1984, a single-cell gel electrophoresis method was developed that allowed measurement of DNA damage in individual cells (1). This technique was later modified and adapted for image analysis, providing greater sensitivity and resolution of subpopulations (2, 3). In this method, single cells are embedded in agarose on a microscope slide, then lysed to remove proteins, exposed to low-voltage electrophoresis, and stained with a fluorescent DNA-binding dye. The amount of DNA that migrates is proportional to the number of DNA breaks present in the cell. This “comet” assay is versatile and able to detect DNA single-strand breaks, double-strand breaks, or cross-links in virtually any cell type, provided that a single-cell suspension can be obtained (3). The method has also been applied to human tumor samples obtained by fine-needle aspiration biopsy (4), a relatively safe and simple procedure, which is applicable to many human tumors. Not only is the comet method sensitive to low levels of DNA damage, but more important, it provides sufficient resolution to detect subpopulations that differ in damage by as little as a factor of 2 (4). Because the tumor cells that survive treatment with a DNA-damaging drug are likely to be those that do not harbor extensive DNA damage following treatment, a DNA damage-based assay is appealing as a surrogate for cell killing.

MATERIALS AND METHODS

**Spheroid Culture.** Chinese hamster V79-171b lung fibroblasts were maintained in exponential monolayer growth by subcultivation twice weekly in Eagle’s MEM containing 10% FBS. SiHa human cervical carcinoma cells and WiDr human colon carcinoma cells were purchased from the American Type Culture Collection and maintained in exponential growth by twice-weekly subcultivation in MEM plus 10% FBS. Spheroids were initiated by seeding 2 × 10⁴ cells/ml into Belco spinner culture vessels containing MEM plus 10% FBS. Spheroids were fed after 3 days and daily thereafter by replacing spent medium with MEM plus 5% FBS supplemented with antibiotics.

Spheroids were exposed to tirapazamine in MEM plus 5% FBS in Belco glass spinner culture vessels. Vessels containing spheroids were equilibrated with 10% oxygen, 5% carbon dioxide, and 85% nitrogen at 37°C for 1 h prior to and during incubation with the drug. Following incubation, spheroids were washed and disaggregated by exposure in an orbital shaker at 37°C for 8–10 min in 0.25% trypsin dissolved in citrate saline. Single cells were centrifuged and resuspended in fresh cold medium. Cells were analyzed for clonogenicity by plating cells in 100-mm tissue culture dishes containing 10 ml MEM plus 10% FCS. Colonies were counted 8–14 days after treatment. Plating efficiencies of cells from V79, SiHa, and WiDr spheroids were 0.64 ± 0.04, 0.66 ± 0.09, and 0.17 ± 0.06 (mean ± SD), respectively. The same population of cells is predictive for cell killing independent of the dose of the drug, hypoxic status, time of exposure, or cell type. Therefore, we tested whether DNA damage by tirapazamine, measured using the comet assay, could be an effective predictor of cell killing in multicell spheroids and tumors in mice and might therefore have potential applicability in the clinic.

**Tumor Cells.** SCCVII squamous cell carcinoma cells were transplanted s.c. over the sacral region of inbred male C3H/HeN mice, approximately 30 g in weight. Tumors were used for experimentation approximately 2 weeks later when they had reached a weight of 350–500 mg. SiHa human cervical carcinoma cells were transplanted s.c. in the back of CB-17 severe combined immunodeficient mice. Cell suspensions were obtained by cutting tumors and washing them in MEM without FBS.

**Comet Assay.** Single-cell nuclear suspensions were obtained using a trypsin (0.25%) and citrate (0.01 M) solution. Cells were pelleted, washed, and re-suspended in a hypotonic solution (0.8% NaCl) for 30 min. Cells were then centrifuged and re-suspended in low-melt agarose (0.8% in 10 mM sodium phosphate buffer, pH 7.4). Single-cell suspensions were dropped onto microscope slides and placed in wells of a horizontal gel electrophoresis apparatus. The specific conditions used in the experiments are described in the figure legend.

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immunodeficient mice. Tumors were used for experimentation approximately 4–5 weeks later when they had reached a weight of 300–500 mg.

Tirapazamine (SR4233) was kindly provided by Dr. Martin Brown (Stanford University). Mice were injected i.p. with tirapazamine from a stock solution of 1–2 mg/ml in PBS. One h after i.p. injection, mice were sacrificed, and tumors were rapidly excised and placed in ice-cold PBS. A single-cell suspension was prepared from the entire tumor by finely mincing the tumor and incubating for 30 min with a mixture of trypsin, collagenase, and DNase as described previously. Cells were then filtered through 30-μm nylon mesh, centrifuged, and resuspended in cold medium. Plating efficiency in MEM plus 10% FCS was 0.28 ± 0.04 for cells from SCCVII tumors and 0.22 ± 0.06 for SiHa cells.

Hoechst 33342 Cell Sorting. During the final 20 min of drug exposure, multicell spheroids were incubated with 1 μg/ml Hoechst 33342 to selectively recover cells from different depths within the spheroid (10). At the end of drug exposure, spheroids were rinsed, disaggregated with trypsin, and sorted into 10 fractions according to the Hoechst fluorescence concentration using a Becton Dickinson FACS 440 cell sorter.

Mice were injected i.v. with 0.1 ml of an 8-mg/ml stock solution of Hoechst 33342 in PBS; then 10 min later, animals were sacrificed, tumors were removed, and a single-cell suspension was prepared for fluorescence-activated cell sorting (11). Cells were sorted into eight fractions according to the Hoechst fluorescence gradient.

DNA Damage Measured Using the Alkaline Comet Assay. Single cells from spheroids or tumors were suspended in ice-cold PBS at a concentration of 2–4 × 10⁶ cells/ml. Then, 0.5 ml cell suspension (10⁶ cells) was placed in a 5-ml disposable tube, and 1.5 ml of 1% low gelling temperature agarose (type VII, Sigma Chemical Co.; prepared in distilled water and held at 40°C) were added and mixed with the cells. Approximately 1.5 ml of this mixture were quickly pipetted onto an agarose-coated, half-frosted microscope slide and allowed to gel. Slides were carefully submersed in an alkaline lysis solution containing 1 M NaCl, 0.03 M NaOH, and 0.2% sarkosyl for 1 h, followed by a 1-h wash in two rinses of 0.03 M NaOH plus 2 mM EDTA before electrophoresis in a fresh solution of 0.03 M NaOH plus 2 mM EDTA at 0.6 V/cm for 25 min. Slides were rinsed and stained for 20 min in 2.5 μg/ml propidium iodide.

Individual cells or comets were viewed using a Zeiss epifluorescence microscope attached to an intensified solid-state CCD camera and image analysis system. For viewing propidium iodide fluorescence, slides were illuminated with green light from a 100-W mercury source using a 580-nm reflector and a 590-nm barrier filter. Individual comets were viewed using a ×25 objective, and 200 or more images were analyzed using a fluorescence image-processing system (2). As the number of DNA strand breaks increased, the amount of DNA able to migrate away from the comet head increased in proportion to the dose (7). The “tail moment,” defined as the product of the percentage of DNA in the comet tail and the distance between the means of the head and tail distributions, and “DNA content,” defined as the total fluorescence associated with an image, were the most informative features (2).

RESULTS

Tirapazamine produces DNA damage and cell killing in SiHa, WiDr, and V79 spheroids, which increase with both time of exposure and drug dose. Representative data are shown for SiHa spheroids in Fig. 1. The average tail moment, which has been shown to be proportional to the number of strand breaks present in the cell (7), is correlated with the surviving fraction in SiHa spheroids, irrespective of drug dose or time of exposure (Fig. 1c). Note that DNA damage must exceed a tail moment of 20 for consistent cell killing to occur.

SiHa spheroids are more sensitive to killing and DNA damage by tirapazamine than either V79 or WiDr spheroids (Fig. 2a), and the relationship between the tail moment and cell killing differs for the three types of spheroids (Fig. 2b). Unfortunately, however, the average amount of DNA damage in a population of cells is not a good predictor of cell killing in a heterogeneous system such as a spheroid or tumor, because a single value could describe a population in which all of the cells show an intermediate level of DNA damage or a population in which some of the cells are undamaged and some of the cells are heavily damaged. The latter situation is the one of concern when measuring tumor response to a cytotoxic agent, because undamaged cells will survive treatment. The amount of DNA damage in cells destined to die is largely irrelevant but will impact on the average tail moment.

Therefore, to predict cell killing based on DNA damage accurately, it is essential to examine the response of individual cells in the comet assay. Heterogeneity in DNA damage is evident in tail moment histograms generated for SiHa spheroids exposed to tirapazamine (Fig. 3, a–d). If one assumes that all of the comets with tail moments ≤20 represent cells that would survive treatment, whereas all of the comets with tail moments >20 would die, then tail moment histograms taken from the experiment in Fig. 1, a and b, accurately predict cell survival (Fig. 3e). However, would the same tail moment discriminator be as successful in identifying survivors among V79 and WiDr spheroids exposed to tirapazamine? Combined results for many experiments are shown in Fig. 4 using three spheroid types, at least five exposure times, and three to four drug doses and applying the same tail moment discriminator of 20. These data also include cell-sorting experiments using the fluorescent dye Hoechst 33342 to separate cells according to position within the spheroid. Within any single experiment, the correlation was generally better (e.g., Fig. 3e); however, in these combined sets, the correlation between measured and predicted surviving fractions is still remarkably good. The slope of the line is 0.92 for all cell lines.

Promising results using multicell spheroids prompted us to perform similar experiments using two solid-tumor models: SCCVII mouse...
squamous carcinoma growing s.c. in C3H mice and SiHa human cervical carcinoma in severe combined immunodeficient mice. Mice were injected i.p. with 4–60 mg/kg tirapazamine, and 60 min later, the mice were sacrificed, tumors were removed and dissociated using enzymes, and single cells were analyzed for clonogenicity and DNA damage. Heterogeneity in DNA damage was also observed in the cells of these tumors (Fig. 5, a–c), consistent with previous experiments using SCCVII tumors (7). A tail moment of ≤20 again provided a useful discriminator for cells that are clonogenic in vitro (Fig. 5d). A complication for exposure in vivo is that the plasma half-life of tirapazamine in mice is estimated to be 15–30 min (12, 13), and the half-time for DNA strand break rejoining is 1–2 h (7). Therefore, it is not surprising that tumor samples removed more than 1 h after injection showed significant DNA repair so that the same discriminator was not accurate in predicting cell survival (Fig. 5d).

DISCUSSION

DNA strand breakage appears to be a useful surrogate end point for cell killing by tirapazamine. However, the average amount of DNA damage sustained by a heterogeneous population of cells is not adequate to predict cell survival. Instead, the proportion of cells that demonstrate insufficient DNA damage for cell killing is a better indicator of cell survival following exposure to tirapazamine.

The tail moment discriminator of 20 was reasonably accurate in describing the responses of three different rodent and human tumor
Predicting Tumor Response to Tirapazamine

Fig. 5. Analysis of DNA damage and survival in SCCVII and SiHa tumors. a–c, representative histograms of SCCVII tumors analyzed for DNA damage 1 h following i.p. injection of tirapazamine. d, the fraction of comets with tail moments <20 was used to predict survival measured by a colony formation assay 14 days later. Triangles with dots, response of tumor cells removed 2 h following tirapazamine injection. For these tumors only, a discriminator of 10 was used to predict survival. The slope (0.94) and 95% confidence limits are shown for all of the data, excluding triangles with dots.

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References

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