PicoGreen Assay of Circular DNA for Radiation Biodosimetry

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We developed a simple, rapid and quantitative assay using the fluorescent probe PicoGreen to measure the concentration of ionizing radiation-induced double-stranded DNA (dsDNA) in mouse plasma, and we correlated this concentration with the radiation dose. With 70 μ l of blood obtained by fingerstick, this 30 min assay reduces protein interference without extending sample processing time. Plasma from nonirradiated mice (BALB/c and NIH Swiss) was pooled, diluted and spiked with dsDNA to establish sensitivity and reproducibility of the assay to quantify plasma dsDNA. The assay was then used to directly quantify dsDNA in plasma at 0–48 h after mice received 0–10 Gy total-body irradiation (TBI). There are three optimal conditions for this assay: 1:10 dilution of plasma in water; 1:200 dilution of PicoGreen reagent in water; and calibration of radiation-induced dsDNA concentration through a standard addition method using serial spiking of samples with genomic dsDNA. Using the internal standard calibration curve of the spiked samples method, the signal developed within 5 min, exhibiting a linear signal ($r^2 = 0.997$). The radiation-induced elevation of plasma DNA in mice started at 1–3 h, peaked at 9 h and gradually returned to baseline at 24 h after TBI (6 Gy). DNA levels in plasma collected from mice 9 h after 0–10 Gy TBI correlated strongly with dose ($r^2 = 0.991$ and 0.947 for BALB/c and NIH Swiss, respectively). Using the PicoGreen assay, we observed a radiation dose-dependent response in extracellular plasma DNA 9 h after irradiation with an assay time ≤ 30 min. © 2015 by Radiation Research Society

INTRODUCTION

Over the last decade, the development of biomarkers for ionizing radiation exposure that are effective at early

response times after irradiation have been investigated in human and animal models. With our increasing awareness of the potential threat of mass casualty radiation events since September 11, 2001, and the Level 7 nuclear accident at Fukushima Daiichi, the U.S. Government has placed a high priority on developing these biomarkers for dose estimation (1, 2). Cytogenetic analysis has been developed for biomonitoring populations occupationally exposed to ionizing radiation (3, 4), and chromosomal aberrations have been accepted as the “gold standard” for evaluating radiation-induced damage in humans (5, 6). However, this approach requires collecting and culturing lymphocytes, which requires several days before a dose estimate can be calculated by highly trained personnel after sample preparation and analysis. Thus, the use of chromosomal aberrations is less amenable to rapid (<15 min), point-of-care biodosimetry methods that would be required for screening large numbers of casualties in the field.

Alternative biomarkers that have the potential to serve as material for rapid, point-of-care biodosimetry assays are being investigated. These efforts include physical dosimetry methods, such as electron paramagnetic resonance (EPR) measurements in teeth (7, 8), and the development of markers that reflect biological responses to ionizing radiation. These biologically based markers of radiation exposure include dose-dependent changes in; red blood cell polyamine levels (9), circulating mRNA patterns (10), serum protein levels (11, 12) and metabolic markers in urine (13, 14). Other types of biodosimetry methods include: radiation-induced damage and repair of DNA (15–17); the consequences of misrepair (4, 18–21) including micronuclei production (4, 18); gene expression profiles, as determined by microarrays (19, 20) and reverse transcription polymerase chain reactions (RT-PCR) (21); and the measurement of dsDNA released into plasma as a consequence of radiation-induced cell death, which is influenced by apoptotic and nonapoptotic mechanisms (21–28). Our group has previously shown that inter-B1 sequences can be used as a trace marker to measure the circulating dsDNA by real-time quantitative PCR in irradiated mice and is a potentially

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FIG. 1. Effect of plasma concentration on PicoGreen fluorescence. Mouse plasma was diluted to 5 different concentrations, as described in the Materials and Methods section. The 100 μ l of reaction mixture consisted of 50 μ l of diluted plasma and 50 μ l of 200 $\times$ diluted PicoGreen dye. Each point represents the mean $\pm$ 1 SD of triplicate measurements.

useful biodosimeter for radiation and other combined toxicities (27). However, this method requires not only restricted environmental conditions to prevent contamination of the samples but also operation by trained personnel, limiting its application for very large events. Therefore, we began to explore simpler and more efficient methods for detecting and quantifying circulating dsDNA concentrations that could be completed in <30 min. We show here that by utilizing the PicoGreen reagent to detect and quantify dsDNA in a variety of matrices, we have developed a method to accurately quantify circulating dsDNA released into plasma after radiation exposure. We propose that this method holds great promise for application in radiation biodosimetry.

MATERIALS AND METHODS

Animals

Male NIH Swiss and BALB/c mice (6–8 weeks old) (National Cancer Institute Mouse Repository, Frederick, MD) were housed in a micro-isolator room at the University of Rochester (Rochester, NY), five animals per cage on a 12:12 h light-dark schedule and fed a standard diet. Care and use procedures were approved by the University of Rochester Institutional Animal Care and Use Committee.

Irradiation

Mice (5–7 animals per dose group) were immobilized in plastic boxes and administered a single dose of total-body irradiation (TBI) using a cesium-137 gamma source at a dose rate of ~ 1.79 Gy/min. After irradiation, mice were returned to their cages until blood was collected.

Preparation of Plasma Samples

A volume of approximately 0.5 ml of blood was collected from each mouse through the lateral saphenous vein into 1.5 ml of

ethylenediaminetetraacetic acid (EDTA)-anticoagulant in Eppendorf Tubes[®] at 9 h postirradiation; after which the mice were humanely euthanized. The blood samples were centrifuged at 5,000 rpm for 3 min at 4°C. Plasma was transferred to new tubes and stored at -70°C .

Optimization of the PicoGreen Assay in Mouse Plasma

PicoGreen reagent concentrate in dimethyl sulfoxide (DMSO) was diluted from 1:25 to 1:400 with Tris-EDTA (TE buffer, pH 8.0). For signal-to-noise, recovery testing and calibration of radiation-induced dsDNA by the standard additions method, plasma samples were spiked with 1 mg/ml of dsDNA stock solution (Promega Corp., Madison, WI).

Plasma from 6 nonirradiated mice was pooled, diluted from 1:1 to 1:10 with double-distilled water (ddH_2O) and added to white 96-well plates (cat no. 762074, Greiner Bio-One, Monroe, NC) as 50 μ l aliquots with or without DNA spiking. Diluted PicoGreen reagent (50 μ l) was then added to the wells. After mixing for 20 s, the plate was read using a Spectra Max M2 Microplate reader (Molecular Devices LLC, Sunnyvale, CA) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm (auto cutoff set at 530 nm). Values were represented as relative fluorescence units (RFU) or DNA concentration (ng/ml).

The variation of background fluorescence and assay signal-to-noise as a function of PicoGreen concentration was calculated as the ratio of the RFU obtained from plasma spiked with 500 ng of DNA/ml to the background RFU in plasma alone.

PicoGreen Assay of Plasma Samples

Individual plasma samples from irradiated or nonirradiated mice were thawed on ice. Plasma was diluted 1:10 with ddH_2O , and a 50 μ l aliquot of the same diluted sample was added to 6 wells (3 sets in duplicate) in white 96-well plates. Each duplicate set was spiked with DNA at concentrations of 0, 100 and 200 ng/ml, and 50 μ l of 1:200 diluted PicoGreen reagent was added to each well and mixed by gently shaking the plate, after which fluorescence intensity was read.

Linear Regression Analysis

Three-point datasets obtained from the standard-additions calibrations were performed; the x-intercept from the analysis was used as the estimate of the plasma DNA concentration in the unspiked sample.

Statistical Analysis

All experiments were done in triplicate, with each triplicate set conducted in duplicate. Mean and standard deviations were calculated from raw data. A Student's *t* test was used to determine the significance of difference. *P* values <0.05 were considered significant.

RESULTS

Optimized Plasma Dilution for High Sensitivity

To reduce interference in detecting DNA stained with PicoGreen by proteins, salts and other plasma components (29), we diluted the plasma to 100%, 75%, 50%, 25% or 10% concentration in water, and 50 μ l of each dilution was mixed with 50 μ l of 1:200 diluted PicoGreen reagent in 96-well plates. We found that Pico Green RFUs were sharply increased in the 10–50% diluted samples (Fig. 1), but plateau when plasma concentrations were greater than 50%.

Recovery of the PicoGreen DNA signal was then assessed in plasma diluted in water (10, 25, 50, 75 and 100%) or TE

FIG. 2. PicoGreen assay sensitivity in plasma. Mouse plasma was diluted to 5 different concentrations, as described in the Materials and Methods section. Serial additions of mouse genomic dsDNA (125, 250 or 500 ng/ml) were added to each of the diluted samples. Recovery of spiked dsDNA was calculated on the basis of a dsDNA external standardization curve and given as the ratio of dsDNA concentration in plasma versus in the TE buffer. Each dataset represents the mean $\pm$ 1 SD of triplicate measurements.

buffer spiked with the same set of serial DNA concentrations (125, 250 or 500 ng/ml). Signal recovery of spiked DNA in TE buffer reached 94.0%, ranging from 92.5–95.9% (Fig. 2). In the plasma dilutions, PicoGreen DNA signal recovery increased as the percentage of mouse plasma decreased from 12% in 100% plasma to 89% in 10% plasma. Therefore, the best PicoGreen DNA signal in TE buffer was in 10% plasma. These results demonstrate that plasma must first be diluted to a ratio of at least 1:10 to prevent interference by protein and other plasma components that reduce the sensitivity of the PicoGreen assay to quantify dsDNA. Because further dilution might have reduced sensitivity to detect DNA, we did not use <10% plasma.

Figure 2 demonstrates that signal recovery did not differ significantly between the three DNA-spiking concentrations

at a given plasma dilution, thereby indicating that at a fixed plasma protein concentration, the PicoGreen fluorescence will increase in proportion to dsDNA concentration, and supports the use of DNA standard-addition for calibrating dsDNA concentration in diluted plasma samples.

Optimized PicoGreen Concentration

The stock PicoGreen dye provided by the manufacturer was diluted to 1:25, 1:50, 1:100, 1:200 or 1:400 with TE buffer. Diluted PicoGreen dye was used to measure 10% plasma with or without a 500 ng/ml dsDNA spike; the final working concentration for samples was one half of the diluted PicoGreen dye stock concentrations. Recovery was calculated based on a known 500 ng/ml DNA standard in TE buffer with the same PicoGreen dye concentration. The best recovery ($103\% \pm 2.4$) was obtained with a working PicoGreen concentration of 1:400 (Table 1). At higher working concentrations of PicoGreen ($>1:100$), background fluorescence increased, resulting in lower DNA recoveries. At a lower working concentration of PicoGreen (1:800), there was lower background fluorescence but insufficient dye to react with DNA, resulting in lower DNA recovery ($77.7\% \pm 5.6$). Therefore, we found the optimal working concentration of PicoGreen was 1:200.

Correlated PicoGreen Signal with DNA Concentration in 10% Plasma Sample

To test the range of linearity in the signal response to DNA concentration in plasma, 10% plasma samples spiked with serial concentrations of DNA were added to equal volumes of 1:100 PicoGreen stock solution. The relationship between RFU and dsDNA concentration ($r^2 = 0.997$, $P < 0.0001$) was linear within the range of 0–800 ng/ml (Fig. 3).

Determined Stability of PicoGreen Signal

The stability of the PicoGreen/dsDNA signal in plasma was then tested in 10% plasma samples spiked with 0, 125, 250 and 500 ng/ml dsDNA. The RFUs were measured at 5, 10, 30, 60 and 120 min. They did not differ ($P < 0.05$) between time points, irrespective of spiked dsDNA amount, indicating that the PicoGreen/dsDNA complex is stable for at least 2 h (Fig. 4).

TABLE 1

Effect of PicoGreen Concentration Background and Signal-to-Noise Ratio of the Assay in 10% Plasma Spiked with DNA

PicoGreen final dilution	10% HP RFU	10% HP + 500 ng/ml dsDNA RFU	Signal-to-noise ratio	Recovery (% $\pm$ SD)
1: 50	3833.5 $\pm$ 108.6	8842.0 $\pm$ 160.1	2.3 $\pm$ 0.03	80.9 $\pm$ 5.1
1: 100	3032.2 $\pm$ 53.5	9130.1 $\pm$ 265.7	3.0 $\pm$ 0.03	88.3 $\pm$ 4.6
1: 200	2485.8 $\pm$ 92.8	9029.2 $\pm$ 105.9	4.6 $\pm$ 0.05	98.2 $\pm$ 3.7
1: 400	1332.6 $\pm$ 40.3	8493.1 $\pm$ 222.2	6.2 $\pm$ 0.04	103.0 $\pm$ 2.4
1: 800	683.6 $\pm$ 39.6	5958.6 $\pm$ 66.6	8.7 $\pm$ 0.06	77.7 $\pm$ 5.6

Note. HP = human plasma; RFU = relative fluorescence units; SD = standard deviation.

FIG. 3. Linearity of PicoGreen assay in modified plasma. Mouse plasma was diluted to 10% with double distilled H₂O, with variable amounts of dsDNA ranging from 70–800 ng/ml added. The PicoGreen signal was linear, and added DNA resulted in a good response, as evidenced by the slope of 11 RFU/ng DNA. The correlation coefficient (r^2) value was 0.998.

Quantified Plasma DNA Based on a Standard-Additions Calibration Method

A 3-level standard-additions calibration method was used for quantifying dsDNA in plasma. Figure 5 shows a plot of 5 calibration datasets for plasma samples taken from NIH Swiss mice 9 h after TBI (0, 3, 5, 7 and 10 Gy). The lines represent the linear regression fit to each of the datasets. The absolute value of the x-intercept calculated from linear regression analysis of each dataset is the estimate of DNA concentration in the diluted (10%) plasma. For the data presented in Fig. 5, units (ng/ml) of DNA concentrations and standard errors calculated from the regression fits are 13.0 (± 5.8), 56.6 (± 6.6), 147 (± 18), 205 (± 50) and 280 (± 61) for 0, 3, 5, 7 and 10 Gy, respectively.

FIG. 4. Stability of PicoGreen dsDNA quantitation assay in diluted plasma. Different amounts of dsDNA ranging from 100–400 ng/ml were added to 10% mouse plasma. The reaction with PicoGreen was recorded at different time points at room temperature in the absence of ambient light. Each dataset represents the mean ± 1 SD of triplicate measurements.

FIG. 5. Calibration of dsDNA concentrations in plasma using standard additions method. Plasma was collected at 9 h after TBI of NIH Swiss mice at doses of 0, 3, 5, 7 and 10 Gy. Diluted (10%) plasma samples were spiked to DNA concentrations of 0, 100 and 200 ng/ml above level in the diluted samples. Data points represent the mean ± 1 SD measured from 6–7 mice per dose point. The lines for each sample dataset represent the linear regression fit to the data. The x-intercept of the regression lines is the estimate of the DNA concentration in the diluted (10%) plasma sample.

Determined Time-Dependent Alteration of Plasma DNA after Total-Body Irradiation

Because radiation-induced DNA release and clearance are in a dynamic balance with time, it was necessary to determine the peak concentration in radiation-induced release of DNA. In NIH Swiss mice irradiated at a 6 Gy dose, which is lower than the median lethal dose (LD_{50}) for this strain, the DNA concentration was measured at different times after irradiation. The PicoGreen assay showed that plasma DNA increased at 3 h postirradiation, peaked at 9 h and then gradually reduced to a near background level at 24 h (Fig. 6A). A similar pattern was observed with BALB/c mice (Fig. 6B). These datasets strongly suggest that the optimum time point for peak levels of DNA based on a single dose (6 Gy) is 9 h after irradiation.

Determined Dose-Dependent Alteration of Plasma DNA after Total-Body Irradiation

A positive correlation between plasma DNA levels and radiation dose was tested in BALB/c mice that were exposed to different doses. In plasma collected at 9 h postirradiation, plasma DNA levels increased monotonically with radiation dose in BALB/c mice (Fig. 7A). A similar result was obtained with irradiated NIH Swiss mice (Fig. 7B). The correlation coefficient was 0.991 for BALB/c mice and 0.947 for NIH Swiss mice. Experiments were repeated in triplicate and showed the same trends. The data strongly support the potential use of plasma DNA concentration measurements as the basis for estimating radiation dose.

DISCUSSION

DNA breakage is the hallmark of radiation damage. Several biomarkers based on measuring damage to DNA or

FIG. 6. Effect of postirradiation time on plasma DNA level. Plasma was collected at different time points after TBI of NIH Swiss (panel A) and BALB/c (panel B) mice at a single dose of 6 Gy. Data points represent the mean $\pm$ 1 SE of the DNA concentration measured from 5–7 mice per time point.

its release from cells have been proposed for estimating radiation exposures (4–6, 15, 16, 18). In this current work, we developed an assay using PicoGreen to quantify the release of dsDNA in plasma after irradiation. The PicoGreen assay is simple, reliable, rapid (under 30 min) and quantitative over a wide range of DNA concentrations. It requires a simple fluorescence reader and uses a straight forward methodology that can be easily mastered. In addition, DNA measured in plasma by the PicoGreen assay is well correlated with real-time qPCR using either SYBR Green or TaqMan probe-targeting β -actin gene ($r^2 = 0.87$ or 0.94 , $P < 0.0001$) (29). The PicoGreen assay has been used to measure plasma DNA levels in patients with cancer and different insults that induce cell apoptosis or necrosis (29, 30). In this is novel study, we explored the assay's ability to determine the extent of radiation-induced release of DNA into plasma.

To demonstrate the capacity of the PicoGreen assay to measure radiation-induced dsDNA, it was first adapted for use in plasma samples. Plasma concentration was found to be the main factor that influenced PicoGreen assay

FIG. 7. Radiation-induced dose effect of plasma DNA level. Plasma DNA concentration in BALB/c mice (panel A) and NIH Swiss mice (panel B) in samples collected at 9 h after exposure to a single dose of TBI as indicated. Data points represent the mean $\pm$ 1 SE of the DNA concentration measured from 5–7 mice per dose point.

sensitivity because recovery of spiked DNA decreased with increasing plasma concentration (Fig. 2), which we found was partly due to increased background fluorescence with increasing plasma content in the diluted samples (Fig. 1). We believe that this nonlinear response in fluorescence intensity with plasma concentration in water was due to the high concentration of proteins in plasma. A variety of factors can affect the signal intensity of PicoGreen dye when bound to dsDNA or in nonspecific binding with plasma components. These factors include salts and proteins (31, 32), which exist in plasma and interfere with the accuracy of assays to varying extents, as has been reported and confirmed by Chen *et al.* (32). Normal adult plasma consists of approximately 6–8% albumin and 1–1.2% IgG (33). A sample with >50% plasma contains >4% albumin, which is expected to limit the ability of the assay to accurately measure changes in radiation-induced DNA concentration in plasma. To avoid this range of insensitivity in the assay, we used plasma at a 1:10 dilution, that reduces the albumin to 0.6–0.8%, much lower than the 2% that has been shown to cause a signal decrease (31). The IgG level, generally at a consistent concentration in normal adults, is

only 0.1% in 10% plasma. This contributes to the background in fluorescence measurements in plasma, albeit at a relatively low and consistent signal level. Solutions to these interferences have been proposed, including the removal of proteins through treatment with protease K or extraction of dsDNA (29, 32, 34). Disadvantages of these approaches include: 1. adding steps to the method and increasing analysis time; 2. possible degradation of DNA during extended sample preparation; and 3. low extraction recoveries of dsDNA (34).

To avoid these problems, we adopted several sample preparation modifications and reagent optimizations. To reduce interference from plasma components, plasma was diluted in water before the PicoGreen reagent was added. Diluting plasma by up to 10% in water provided the optimal balance between decreasing the interference of fluorescence generated by nonspecific interactions of the PicoGreen dye with plasma components (Fig. 1) and excellent recovery of fluorescence signal from PicoGreen bound to DNA (Fig. 2). Because signal intensity is positively related to sensitivity of the assay, we examined other methods to optimize the detection of the signal from PicoGreen binding to DNA. This was achieved through optimizing the PicoGreen concentration in diluted samples. In plasma diluted to 10%, detection sensitivity increased with decreasing PicoGreen dye concentration (Table 1), with an optimum working PicoGreen concentration in samples of 1:400. After implementation of 10% dilution of plasma and optimized PicoGreen dye stock concentration (1:200), good linearity in the signal response with increasing DNA concentration was obtained within the range of 0–800 ng/ml (Fig. 3). Because PicoGreen's dye signal varies with time, we examined the signal stability in plasma spiked with dsDNA. Using this methodology, we found there was minimal variability of dsDNA signal during a 2 h period (Fig. 4). Notably, the presence of different proteins and other components in plasma vary with the age, sex or health of an individual, as well as with food intake and other factors.

PicoGreen, an N-bis-(3-dimethylaminopropyl)-amino residue, carries three positive charges (35) that likely contribute to the high binding affinity for dsDNA. These molecules share the property of binding selectively to dsDNA by intercalation, resulting in an increase in fluorescence emissions. The PicoGreen excitation/emission is 390/505 nm. Hemolysis takes place *in vivo* due to the rupture or lysis of red blood cells within circulation and *in vitro* either in the blood collection tube or during collection. Red blood cells are susceptible to oxidative stress and become hemolyzed when oxidative stress becomes too high (36, 37). Ionizing radiation-induced oxidative stress leads to an increase in red blood cell lysis or rupture *in vivo* and *in vitro*, thereby releasing hemoglobin into plasma. Because hemoglobin has a wide absorption spectrum of 300–800 nm, it can quench the fluorescent signal of PicoGreen fluorophores (excitation/emission 390/505 nm) in blood

plasma (38). These variations in plasma components result in variances in background fluorescence of plasma samples that could confound the calibration of DNA concentration in samples using external calibration methods. Therefore, a standard additions calibration method based on two spiked DNA samples in addition to the unspiked sample was used to calculate plasma DNA levels (Fig. 5), instead of using an external standardizations approach. This calibration method provided more accurate assessments of DNA concentration than the external calibration method.

Application of the PicoGreen assay as a potential biodosimeter takes advantage of the acute cytotoxicity that occurs in response to total-body and partial-body irradiation. Dying cells are likely the major sources of DNA released into plasma (32, 34), although all source(s) of circulating DNA are unknown. Using a less sensitive assay, Belokhvostov *et al.* found that plasma DNA levels were altered after high-dose irradiation in a rat model (28). Comparing our results using PicoGreen with previously published methods (PCR of the B1 gene sequence not shown here) demonstrates the strong correlation between the data shown here (for PicoGreen) and PCR of the B1 gene sequence; further, our data show a time and dose dependence that peaked at 9 h postirradiation, that provides a dose response in the clinically relevant range of 2–10 Gy (27). The potential of the PicoGreen assay as a biodosimeter is further supported by experiments performed in additional mouse strains of different backgrounds (Figs. 6A and B, 7A and B).

After a mass casualty nuclear disaster, effective medical triage has the potential to save tens of thousands of lives. Given the multisystem nature of radiation injury, it is unlikely that any single biodosimetry assay can be used as a standalone tool to meet the surge in capacity required and is both rapid and accurate. Major factors we considered include the type of sample required, dose detection limit, time interval when the assay is biologically feasible, time for sample preparation and analysis and ease of use. Currently, the existing and developing biodosimetry assays have their advantages and limitations. The PicoGreen assay can provide a simple, rapid, and quantitative screen for relatively high doses of radiation in mice. Therefore, for the PicoGreen assay, further research is necessary to investigate lower dose, partial body exposure, source cells of circulating dsDNA, and applicability to humans in a wide radiation dose range.

CONCLUSIONS

In summary, we have presented a simple, fast, reliable and sensitive method to directly quantify dsDNA levels in mouse plasma. Our results have shown that plasma dsDNA levels measured directly by the PicoGreen assay after dilution to 10% have a specific time course after radiation exposure and increase with radiation dose. The results can be obtained in less than 30 min and correlate well with PCR

of the B1 gene sequence already used in our laboratory. With our methodology, accurate assessment of dsDNA levels requires specimen spiking with dsDNA to internally calibrate the system, thereby avoiding overestimation and underestimation of plasma dsDNA levels.

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