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Strand Break Formation in Plasmid DNA Irradiated in Aqueous Solution: Effect of Medium Temperature and Hydroxyl Radical Scavenger Concentration

HIROYUKI TOMITA, MICHIAKI KAI, TOMOKO KUSAMA and YOSHIRO AOKI

Department of Radiological Health, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan

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plasmid DNA/strand break/aqueous solution/temperature/lifetime of hydroxyl radical

Plasmid pBR322 DNA (4363 base pairs) in aerobic aqueous solution was irradiated with ^{60}Co γ-radiation. The change of diffusion coefficients (D) of chemical species, rate constants (k) of radical-DNA interaction and solubilities of O_2 in water cannot be ignored when a temperature varies more than a few tens of centigrade. It is important to examine the variation of the yields of DNA strand breaks as a function of temperature in order to analyze the mechanisms of DNA strand breaks from the chemical point of view. Hence, we observed the change of the yield of strand breaks with temperatures between −20 and 42°C by agarose gel electrophoresis. We also observed the change of the yield of strand breaks with the concentration of OH scavenger (Tris) from 1 mmol dm^{-3} to 100 mmol dm^{-3} and summarized it with previous experiments. This summarization indicated that the order of the lifetime of OH radical in cellular environment is several nanosecond. This value is consistent with the measurement of the lifetime of 8.7 nanosecond for OH radical in mammalian cell (Roots, R. and Okada, S. (1975) Radiat. Res. 64, 306–320).

INTRODUCTION

It is widely accepted that many biological effects of ionizing radiation are a consequence of DNA damage. The quantitative analysis of DNA damage induced by low-LET radiation is important to progress the research of radiation biology and protection. DNA strand breaks induced by low-LET radiation in the cellular environment are mainly caused by reactions with diffusible hydroxyl radicals (OH) generated from water radiolysis[1,2], but cellular systems are too complicated to analyze mechanisms of DNA strand breaks in detail. Small circular DNA are more useful for studying the effect of various physico-chemical reactions on DNA. Above all, plasmids are a convenient model system since they are of a well defined size, and the detection

富田裕之，甲斐倫明，草間朋子，青木芳朗：東京大学医学部放射線健康管理学教室，東京都文京区本郷7-3-1 〒113

of strand breaks is readily accomplished by gel electrophoresis. Several experiments using plasmid were reported[2-8]. However, these reported values of DNA strand breaks were mostly based on only three pieces of experimental data. The previous data were thought to be limited and insufficient for comparing them with analytical calculation.

The decrease in the mobility of molecules followed by a decline in the rate constant of chemical reactions is an obvious consequence of cooling. The temperature dependence of diffusion coefficients (D) of chemical species, rate constants (k) of radical-DNA interaction and solubilities of O_2 in water can be estimated by Arrhenius equation. For example, the k of the self-recombination of e_{aq}^- ($e_{aq}^- + e_{aq}^- -> H_2 + 2OH^-$) at 42°C is about ten times higher than at −10°C (the activation energy is 22.6 kJ/mol[9]). These changes of D and k reflect molecular-level interactions. From the chemical point of view, variation of the yields of DNA strand breaks as a function of temperature cannot be ignored in analyzing the mechanisms of DNA strand breaks. For this reason, we examined temperature effects on the OH-attack to DNA in liquid-phase water (−20~42°C). These temperatures were closely related to the activity of creatures. The competition kinetics between DNA and OH can be altered by changing the ratio of OH-radical-scavenger concentration and DNA concentration. Thus, we also studied the effect of the concentration of OH-scavenger and compared it with the previous studies[2,3,7,8,10,11] In these studies, radical scavengers were added prior to irradiation. This is because the competition kinetics between DNA and OH assumed to be constant throughout radiation exposure. We checked this assumption by a preliminary experiment that radical scavenger (Tris) was injected in the mid-point of irradiation period. If the competition are kept constant throughout exposure, just half number of strand breaks are observed.

We have been studying the mechanisms of OH-induced DNA strand breaks by computer simulation using track-structure approach of microdosimetry[12]. This experimental data of plasmid DNA will give us useful information to refine our computer simulation of physico-chemical process of the radiation-induced DNA strand break.

MATERIALS AND METHODS

DNA and Irradiation

pBR322 plasmid DNA (4363 base pairs, molecular weight about 2.8×10^6 dalton) was obtained from GIBCO BRL. The plasmid DNA was suspended in TE solution. Solutions of

Table 1. Substances used to temperature experiment

Substance	Mixing amount [g] (to 100 g ice)	Temperature [°C] (±0.5°C)
$MgSO_4 \cdot 7H_2O$	51.5	−3
$Na_2S_2O_3 \cdot 5H_2O$	67.5	−9
NH_4Cl	25	−13
$(NH_4)_2SO_4$	62	−16
NaCl	22.4	−20

plasmid DNA (29.75 μg/cm^3) were irradiated with ^{60}Co γ-ray aerobically in 500 μl polypropylene tubes immersed in an ice bath at lower temperatures (−20, −16, −13, −9, −3, and 0°C; Table 1) or in a warm bath at higher temperatures (20, 25, 36, and 42°C) in the presence of OH scavenger (Tris, EDTA). We changed the concentration of Tris from 1 mmol dm^{-3} to 100 mmol dm^{-3}. The temperature of the bath was measured by thermocouple-thermometer (Techno Seven Co) both before and after irradiation. Solutions were made from water which was purified by filtering double distilled water through a MilliQ millipore system and solutes of analytical reagent grade. The values of the rate constants k for the reaction of scavenger with OH which were used for the calculation were: Tris[13], 1.5×10^9 dm^3 mol^{-1} s^{-1}; EDTA[3], 1.2×10^9 dm^3 mol^{-1} s^{-1}. The irradiated doses were 6, 12.5, 25, 50, and 100 Gy. The dose-rates varied from 5.62 to 45.45 Gy/min. Within these ranges, the dose-rate had no effect on the yield of damage. The dose-rates were determined by Fricke dosimetry.

Electrophoresis

Immediately after irradiation, the samples were mixed with 1/10 vol of a loading buffer [5% glycerol containing 0.25% Bromphenol Blue]. Supercoiled, open circular and linear forms of the plasmid were separated by agarose gel electrophoresis (Mupid-2, Cosmo Bio). The gels contained 1.4% agarose in TAE buffer (40 mmol dm^{-3} Tris-acetic-acid, 2 mmol dm^{-3} EDTA, pH 8.1) and were run for 5 h at 4.13 V/cm at 4°C. Gels were photographed using a red filter, and the ethidium bromide fluorescence bands were quantified by scanning the negatives with a PDI-1D gel analysis scanner (Toyobo Co). The amount of DNA applied to the gel was 0.19 μg on average, so that linear relationship between the amounts of DNA and the peak area multiplied by the optical density of film was obtained in our experiment, which is shown in Figure 1. To account for the lower binding constant of ethidium to supercoiled plasmid, relative to the open circular and linear conformations, intensities of supercoiled bands were multiplied

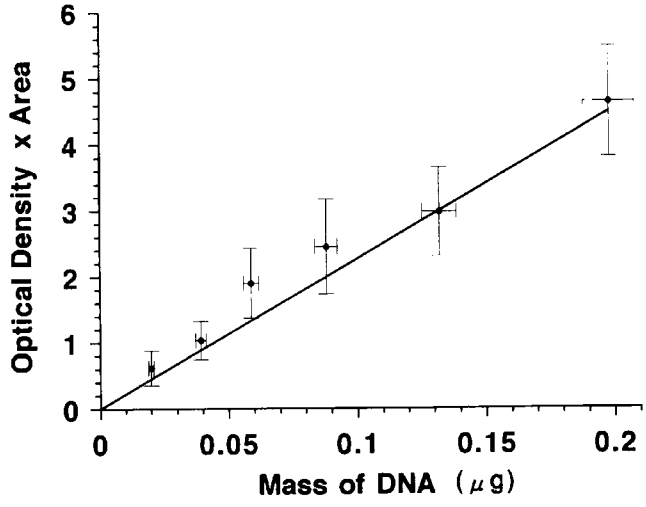

Fig. 1. Relationship between fluorescence intensity enhanced by EthBr and mass of plasmid pBR322 DNA

by factor 1.45 which was the average of two reported values for plasmid pBR322; 1.4[7], 1.5[5]. The average number (x) of SSBs and of the average number (y) of DSBs per plasmid molecule were determined by the following equation[4].

$$x = \ln[(1-L)/S]$$

$$y = L/(1-L)$$

where S and L are the fractions of detectable molecules corresponding to supercoiled and linear form of the plasmid, respectively. To check the consistency of our data, DNA samples were irradiated by the same condition and the number of strand breaks were measured once a month and compared with each other through the period of our experiment.

Timing of adding radical scavenger to sample

10 μl of Tris (1,10,100 mmol dm^{-3}) was added to 40 μl of the DNA medium (29.75 $\mu m/cm^3$

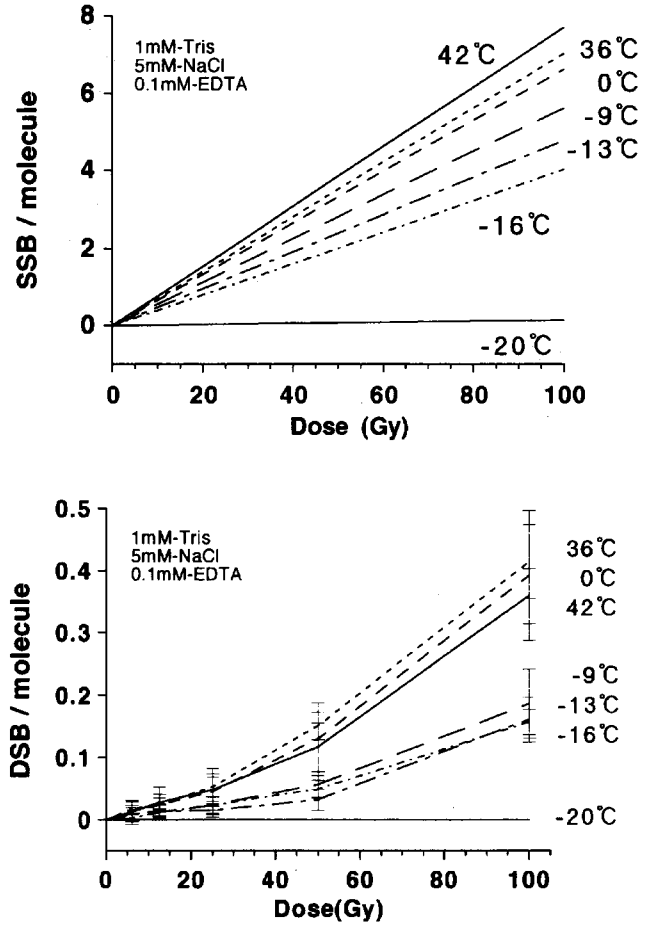

Fig. 2. The effects of temperature on the yields of SSBs and DSBs induced by ^{60}Co γ-ray in dilute aqueous solution (29.75 $\mu g/cm^3$ DNA, 1 mmol dm^{-3} Tris, 5 mmol dm^{-3} NaCl, 0.1 mmol dm^{-3} EDTA)

DNA, 1 mmol dm^{-3} Tris, 5 mmol dm^{-3} NaCl, 0.1 mmol dm^{-3} EDTA) at 10sec-, 2sec-before, or at the middle, or at 2sec-later, 10sec-later of the 50 Gy-exposure. Consequently, when Tris was injected in the middle of exposure, just half number of strand breaks were produced. The competition kinetics between DNA and OH were verified to be constant throughout radiation exposure. Hence, we added radical scavenger (Tris) prior to irradiation.

RESULTS

The effcts of temperature of medium

The relation between the yield of radiation-induced strand breaks and temperature is shown in Figure 2. The number of SSBs increased linearly with the dose. The induction of DSBs was found to be a linear-quadratic function of the dose. A quarter of the samples in this study were observed to be frozen at −16°C and all samples were frozen at −20°C. The yields of SSBs of frozen samples at −16°C was the same as those of −20°C (no DSB was observed), so that frozen data of −16°C (3 data) was not used for this plot. Each curve was obtained from twelve experiments (except −16°C), and the error-bars of SSBs were omitted for the simplicity of the figure. Figure 3 shows the effect of temperature on D_{37} of the yield of SSBs. The D_{37} doses were constant between −3°C and 36°C, and increased with decreasing the temperature below −3°C, and then it was amplified 30 times when the sample was frozen. The induction efficiencies for SSBs and DSBs as a function of temperature are shown in Figure 4. The yield of DSBs was not a linear function of radiation dose, so that induction efficiency of DSBs differs when calculated by either 50 Gy-data or 100 Gy-data. The yields of SSB and DSB induction were estimated 10^{-8}/Gy/dalton and 10^{-10}/Gy/dalton, respectively.

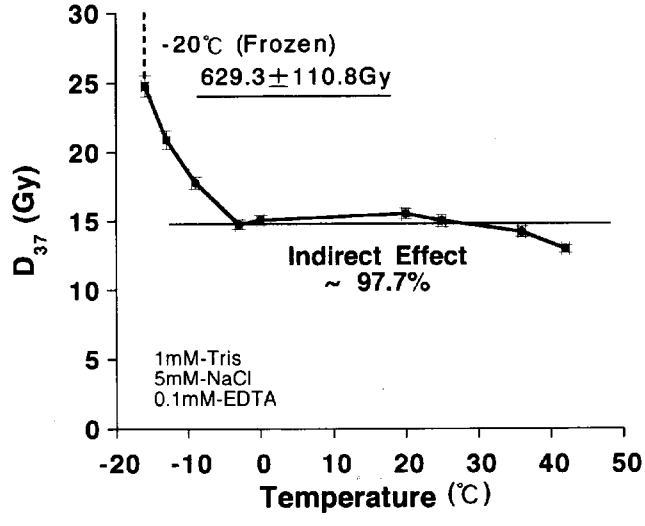

Fig. 3. The effects of temperature on D_{37} for plasmid pBR322 DNA in dilute aqueous solution (29.75 $\mu g/cm^3$ DNA, 1 mmol dm^{-3} Tris, 5 mmol dm^{-3} NaCl, 0.1 mmol dm^{-3} EDTA)

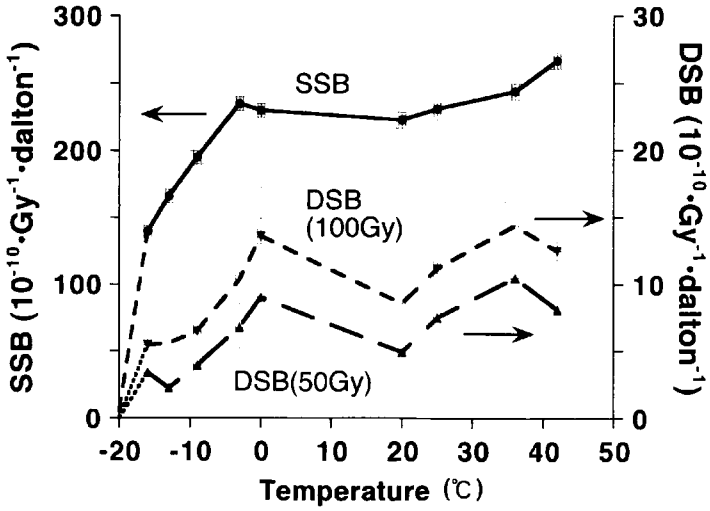

Fig. 4. The effects of temperature on the induction efficiencies for SSBs and DSBs by ^{60}Co γ-irradiation in dilute aqueous solution (29.75 μg/cm^3 DNA, 1 mmol dm^{-3} Tris, 5 mmol dm^{-3} NaCl, 0.1 mmol dm^{-3} EDTA)

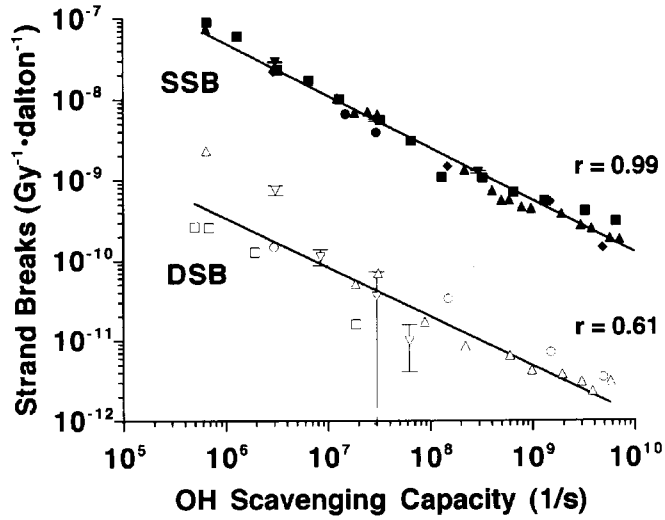

Fig. 5. Yield of single-strand breaks (SSBs) and one-hit double-strand breaks (aDSBs) after ^{60}Co γ-irradiation of small circular DNA. (▲plasmid pBR322; ref.2, ■plasmid pUC18(2686 bp), pBR322; ref.7, ◆SV40(~ 5300 bp); ref.10, ●pBR322; ref.11, ▼pBR322; our data, △pBR322; ref.2, □pBR322; ref.4, ○SV40; ref.10, ▽pBR322; our data). The correlation coefficients (r) are also shown.

The effects of the concentration of OH scavenger

A double-logarithmic plot of the SSB yield versus scavenging capacity (σ) shows that the points fall approximately on a straight line (Figure 5, solid symbols) and the fitted curve was

(SSB)$=3.79\times10^{-4}\cdot\sigma^{-0.648}$. The yields of DSBs with linear and quadratic dependence on the dose were determined by plotting the total number of DSBs per unit dose versus the dose and extracting the values of the yield for αDSBs from the intercept and those for βDSBs from the slope of the resulting straight line[14]. A double-logarithmic plot of the αDSB yields versus σ also showed that they decreased almost linearly (Figure 5, open symbols) and fitted curve was (αDSB)$=1.63\times10^{-6}\cdot\sigma^{-0.613}$.

DISCUSSION

Dose-effect relationship

Our results showed a linear relation between the SSB production and the dose, and linear-quadratic relation between DSB production and the dose. These results were consistent with earlier studies of small circular DNA[2,14–16]. On the contrary, linear dose-effect relation of DSBs in a cellular environment was reported[17,18]. This linear relation was determined by pulsed-field gel electrophoresis (PFGE) carefully calibrated with Iodine-125 or restriction enzyme *Not*I. This contradiction might be due to high scavenging capacity in cellular environment. The reported induction efficiencies of DSBs were 10^{-11} to 10^{-12}/Gy/dalton in cellular environment[17,18]. These values were equivalent to the scavenging capacity of 10^9 s^{-1} in small-circular-DNA experiments (Figure 5). The lifetime of OH radical is the inverse of the scavenging capacity of the solution, so that OH-radicals in cells are probably scavenged in nanoseconds. Roots and Okada measured the lifetime for OH-radical in mammalian cell[19]. This value (8.7 nanosecond) is consistent with the above estimation. Figure 2 shows that a 100 Gy-exposure was needed to observe a non-linear dose-effect relationship of DSBs even though the solution of dilute OH-radical scavenger (scavenging capacity is$\sim10^6$ s^{-1}). Thus, a exposure of several thousands of Gy may be needed to observe a non-linear dose-effect relation of DSBs in cellular environment. This is because the concentration of OH-radical scavenger in cells (scavenging capacity is$\sim10^9$ s^{-1}) is about one thousand times higher than our experimental condition (scavenging capacity is$\sim10^6$ s^{-1}). The induction of SSB is thought to follow one-hit kinetics while that of DSB thought to shift gradually from having a major quadratic (two-hit) component at a very low scavenging capacity to nearly a pure linear (one-hit) for capacity$>10^7$/s[10]

The contribution of the indirect effect of radiation

If only "direct effect" of radiation induces strand breaks in frozen-phase, 98% of the strand breaks were estimated to be produced by "indirect effect" in our experiment, from the result of D_{37} of the yield of SSBs (Figure 3). This experimental result showed a good agreement with our previous calculation ($98.5\pm2.5\%$)[12]. The above assumption is oversimplified one and indirect effect cannot be diminished in frozen phase[20]. Moreover, the relative contribution to the strand breaks of this "direct" and "indirect effect" is still a basic problem in radiation biology. However, DNA strand breaks were formed principally by "indirect effect" in the solution of dilute OH-radical scavenger.

The effects of medium temperature on DSB induction

Simple relations between DSB induction and temperature were not observed in our experiment. The minimum of DSB induction between −3°C and 42°C was measured at 20°C. The induction efficiency of DSBs at 20°C was six tenth of that of 36°C (Figure 4). Jones *et al.* reported that when the DNA was held at low temperature (<2°C) before and during electrophoresis, the measured yields of SSBs and DSBs were twofold less than in samples exposed to room temperature[21]. In contrast, if the DNA was incubated at 37°C overnight, the yield of DSBs increased twofold over the room temperature assay, while the SSB yield increased only to a small extent (≦20%)[21]. However, we maintained all DNA samples at low temperatures before (≦2°C) and during (≦4°C) electrophoresis. So that, the effect of postirradiation-holding-temperature was irrelevant to our experiment. Collins suggested that DNA involving unrepaired lesion (e.g. SSB) had a high sensitivity of DSB[22]. When non-irradiated plasmid DNA was analyzed by agarose gel electrophoresis, about 10% of them were observed in open circular form (no linear form was observed). Even a control DNA has SSBs and these initial-SSBs were thought to be left unrepaired in our experiment, because no repair enzymes were involved. The number of initial-SSB probably influenced the outcome, but no correlation was found between the ratio of open circular form of control plasmid and the number of radiation-induced DSBs. In order to progress the understanding of this phenomenon, the experimental work of neutron scattering[23] for observing the change of clustering structures of H_2O molecules might be suggestive. Moreover, many authors[e.g.23,24] have detected anomalies in the temperature dependence of some physical properties of water at these temperatures (15~30°C) and DSB was more sensitive than SSB to the change of temperature[21].

The ratio of SSB/DSB

The observed ratio of SSB/DSB was from 23.9±4.8(based on 100-Gy data) to 40.4±14.9 (based on 50-Gy data) and almost independent of temperatures in our experiment. To explain these ratios, it is thought that two independently introduced SSBs in opposite strands results in a DSB. Several maximum distances between two SSBs that produce one DSB had been estimated by the curve fitting of the data of small circular DNA. The reported distances were widely distributed between 10 to 60 base pairs[2,16,25,26]. However, 5~10 base pairs seem to be a more appropriate distance. These values were suggested by direct measurements by ESR[27], disintegration of ^{32}P atoms incorporated in λ phage DNA[28] and electron migration study in aqueous solution[29].

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