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ATOMIC FORCE MICROSCOPY INVESTIGATION OF RADIATION-INDUCED DNA DOUBLE STRAND BREAKS

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Abstract

We have used atomic force microscopy (AFM) to study radiation-induced DNA double strand breaks. Double-stranded plasmid DNA was irradiated with 18-MeV electrons in aqueous buffer, using a medical linear accelerator. Doses of 50, 100, 150, and 200 Gy were delivered to DNA samples, and atomic force microscopy was used to measure the length of each DNA fragment. From these measurements, we obtained the average length of the irradiated DNA for each sample and found a linear-quadratic relationship between the average length and radiation dose.

Key Words: Atomic force microscopy, DNA, ionizing radiation, double strand breaks, linear-quadratic, fragmentation, aqueous solutions.

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Introduction

It is generally accepted that DNA is the critical target for ionizing-radiation-induced biological effects. In particular, DNA double strand breaks (DSB) have been identified as the lesions most responsible for the lethal effects of ionizing radiation. As a consequence, much effort has been made to explore the relationship between the DSB and radiation dose. However, the exact nature of the relationship is still controversial. A linear dose response has been reported following exposure of DNA up to 250 Gy (Corry and Cole, 1968; Lehmann and Ormerod, 1970), while others have reported a linear-quadratic relationship (Freifelder and Trumbo, 1969; Radford, 1985; Siddiqi and Bothe, 1987).

Four methods have been commonly used for the measurement of DNA strand breaks (Michael, 1991). Neutral gradient sedimentation provides a direct measurement of the molecular weights of DNA fragments. A limitation of this method is its low sensitivity. Neutral filter elution offers improved sensitivity, but its physical basis is poorly understood. In addition, the elution process itself causes a significant amount of DNA fragmentation. Two-dimensional gel electrophoresis is capable of separating large DNA fragments by way of their molecular weight, and thus can also be applied to low dose range. However, the physical basis of this technique is not understood well, and this affects quantification. Premature chromosome condensation offers good sensitivity but it scores only 10-15% of the DSBs.

Because these problems exist with the various existing methods for DNA strand break measurements, Michael (1991) asked in his review paper: "Will DNA damage measurements ever be sensitive enough to validate models?" He indicated that a better understanding of the more sensitive techniques was needed before they can be used confidently to support, or disprove, the validity of models linking DNA damage to biological effect.

A different approach has been taken by Baverstock (1985) to measure DNA double strand breaks. He used electron microscopy to directly measure the length of DNA fragments induced by radiation and compared his

results with a theoretical estimation. This method has the merit of directly visualizing each DNA fragment with consistent resolution. However, technical limitations in sample preparation made some biologists skeptical of the information acquired. Only after many years of persistent effort in the development of specimen preparatory techniques, did electron microscopy finally become a widely accepted method in biology.

The invention of atomic force microscopy (AFM) (Binnig *et al.*, 1986) created a new horizon for biological research, and for the study of DNA molecules and their interaction with other agents. As with the electron microscope, one can directly visualize DNA molecules with a high resolution to a few nanometers (Hansma and Hoh, 1994). The physical basis of this technique is clear and well understood and the sample preparatory procedures are simple. Since its invention, various techniques for sample preparation and imaging have been established and have matured to the extent that the artifacts associated with earlier experiments can now be addressed, corrected, and eliminated. AFM has found wide applications in physics, chemistry, biology, and other disciplines. In particular, AFM has been used to image DNA routinely in both air and aqueous solutions, and to observe enzyme-DNA interaction *in vitro*, sometimes with a resolution high enough to differentiate the DNA double helical turns (Hansma and Hansma, 1993). Here, we report the first experiments using AFM to measure radiation-induced DNA double strand breaks in aqueous solutions. Our results yield a linear-quadratic dose response relationship for doses up to 200 Gy.

DNA Sample Preparation

Double-stranded pUC19 DNA (2742 bp, New England Biolab, Beverly, MA) was diluted in 10 mM Hepes and 1 mM $MgCl_2$ buffer to a concentration of 5 ng/μl. For the first round of experiments, a total volume of 1000 μl was divided into five smaller volumes, each containing 200 μl DNA. Four samples were used for irradiation, and one was kept as a control.

For electron irradiation, we used a medical linear accelerator (Varian 2100 C/D, Varian Associates, Inc., Palo Alto, CA). A 10 cm $\times$ 10 cm cone was used to control the radiation field. In this field size, the electrons have uniform dose distribution. The electron energy was set at 18 MeV, which is the highest achievable energy with this linear accelerator. The DNA was irradiated at room temperature. The pulsed dose rate was set at a nominal 1000 cGy/min. Doses of 50, 100, 150, and 200 Gy were delivered to four DNA samples. The experiment was repeated under identical conditions with another set of four DNA samples.

For AFM imaging, we used a volume of 1 μl of the DNA samples and deposited it onto freshly-cleaved mica. The sample was rinsed with distilled water to remove the excess DNA fragments, and then dried with N_2 gas.

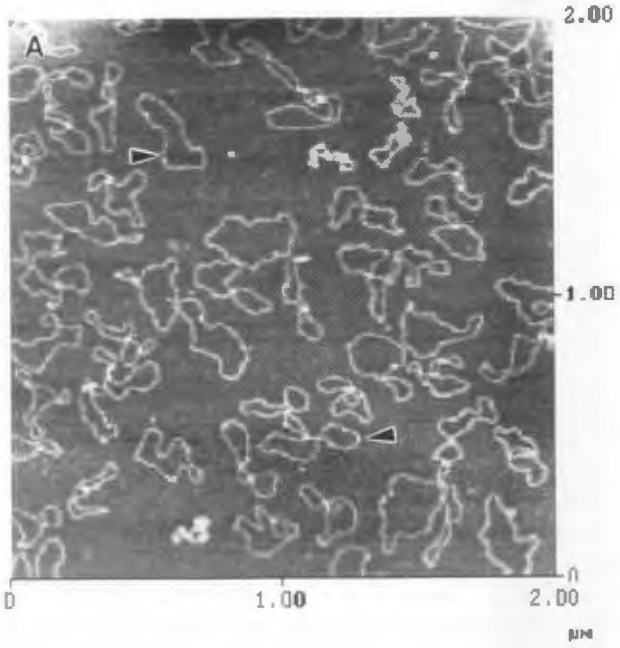


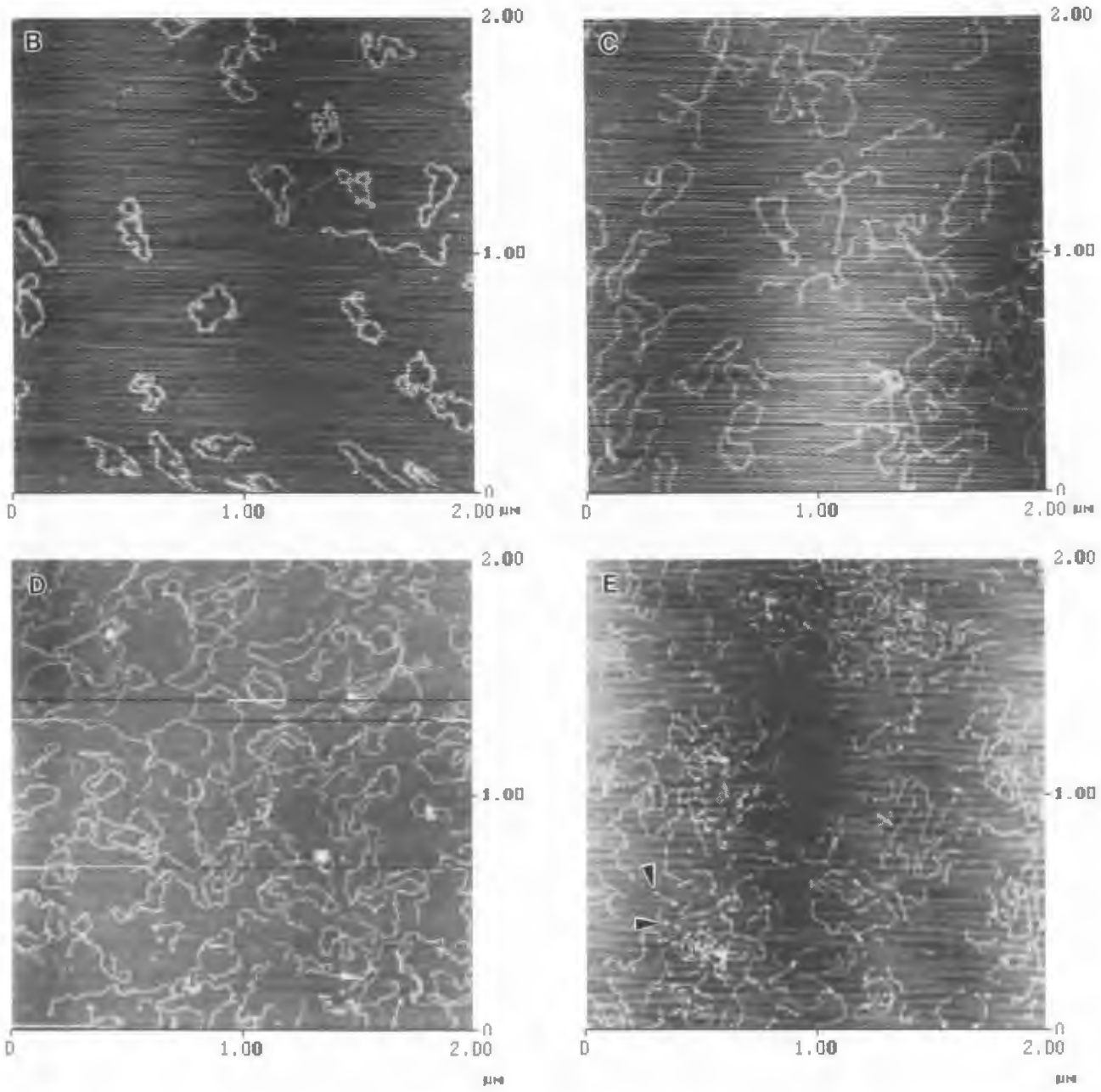
Figure 1 (A above, B-E on the facing page). Representative AFM images of control and irradiated DNA samples. (**A**) Control sample. Over 99% of the plasmids are in intact circular forms. (**B**) 50 Gy, (**C**) 100 Gy, (**D**) 150 Gy, and (**E**) 200 Gy irradiated samples. As shown clearly, the fragmentation of DNA molecules increases monotonically with the increasing dose. The arrowheads identify the kinks and blunting as mentioned in the text.

AFM imaging was performed using a NanoScope-3 (Digital Instrument, Santa Barbara, CA) in its tapping mode in air at room temperature. The cantilevers used are fabricated from Si, the length of which is about 125 μm. The spring constants of the cantilevers are 16-88 N/m. The scan rate was set at 4 Hz/s.

Results

For each sample, we collected 15 images under the conditions specified above. A representative image for each sample is shown in Figure 1. For the control sample shown in Figure 1A, over 99% of the DNA molecules are in complete closed circles. The background level of DSBs determined for our DNA preparation is less than 1%.

On average, the number of DNA fragments in each scanning field (size 2 μm $\times$ 2 μm) varied from about 30 to 48. The lengths of DNA fragments were measured



using the image analysis software incorporated in the NanoScope-3. A large curved fragment was measured by following its contour length in a piecewise manner; each piece was treated as a straight line. The total length of the contour is thus the sum of the length of the straight lines. The uncertainty introduced could be minimized by dividing a contour into a large number of intervals. For our measurement, it is less than 2%.

For the control sample, over 99% of the DNA molecules analyzed were in intact circular forms. The average length of the molecules was measured to be 850 nm with a standard deviation (SD) of 43 nm.

For the 50-Gy irradiated DNA sample, over 88% of the fragments were distributed in the range from 750 to 850 nm, yielding an average length of 790 nm. Fragments as short as 87 nm were also observed. Because of the wide range of fragment size, the SD was found to be 137 nm.

For the 100-Gy irradiated sample, 67% of the fragments were distributed in the range from 750 to 850 nm, and 23% of the fragments were distributed in the range from 143 to 750 nm. The average length is 690 nm, and the SD is 216.

For the 150-Gy irradiated sample, the average

length is shifted to 400 nm. However, the largest number of fragments was centered around 200 nm, accounting for about 40% of the total fragments. About 20% of the fragments were distributed around 840 nm. Such a distribution yielded a SD which was more than 50% of the average.

For the 200-Gy irradiated DNA sample, the average length of the fragments was reduced to 200 nm. The largest number of fragments (36%) was distributed in the 50 to 150 nm range, and 32% in the 150 to 250 range. The SD was 130 nm.

The above data were then used to construct a mean fragment length versus dose curve. The error bar for each data point was the standard error of mean (SE), which is the SD divided by the square root of the number of fragments analyzed for each sample. The SE is a measure of the closeness of the measured mean to the true mean when the number of fragments analyzed is infinite. The results are displayed in Figure 2. A linear-quadratic equation was found to fit the data best, although a pure quadratic fit (no linear term) could not be ruled out.

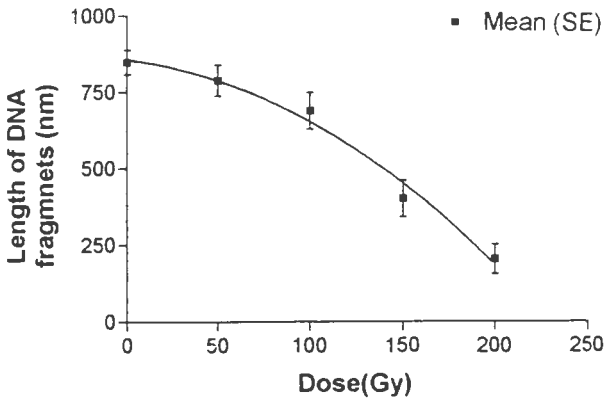


Figure 2. Dose-response data for DSB induction in pUC19 plasmid DNA in aqueous solution. The vertical axis is the length of fragments in nm and the horizontal is the dose in Gy. The data presented are the average length of DNA fragments at each dose. The error bars are standard error of mean (▪). A quadratic polynomial in the form $a + bx + cx^2$ gives the best fit to the data.

Discussion

The exact relationship between the DSB and radiation dose is still a matter of much controversy. Both linear and linear-quadratic relationships have been reported for DNA in cellular environments and in aqueous solutions. More recently, Siddiqi and Bothe (1987) used laser light scattering to measure the molecular weight of calf thymus DNA fragments induced by Co-60 gamma-rays, and found a linear-quadratic relationship in the selected range of radical scavenger (phenol) concentrations. Krisch *et al.* (1991) have also explored the DSB dose-response relationship for DNA in aqueous solutions. They used electrophoresis to measure Cs-137 gamma-ray induced SV40 DNA double strand breaks in dilute buffer at various radical scavenger (Tris) concentrations. They also found a linear-quadratic relationship for DSB production at low Tris concentrations, but almost a pure linear relationship at high Tris concentration. The shift from linear-quadratic to linear increases monotonically with the scavenger concentration levels. They suggested that their findings may clarify some of the controversy reported in earlier experiments, namely, that the nature of the dose-response is dependent on OH radical scavenger concentration.

Our technique using AFM has the merit of providing complete fragmentation distribution. Each DNA fragment was measured individually. The high resolution of AFM can limit the uncertainty in the measurement to a mere 2%. The linear-quadratic relation obtained was the result of the best fit to the average length of fragments using the equation $a + bx + cx^2$, which was also used by the two groups mentioned above to fit their data. In our fit, the coefficient of the linear term, b, is -0.805, and the quadratic term, c, is -0.013. The goodness of fit is $R^2 = 0.986$. The ratio of the two coefficients, b/c, is 54, which is the on the same order of magnitude as that obtained by Siddiqi and Bothe at zero scavenger concentration, but a order of magnitude higher than the value obtained by Krisch *et al.* (1991) at Tris concentration 0.1 mM, the lowest scavenger concentration they tested. Such a discrepancy is not surprising since they controlled the experimental condition so that only one DSB could be induced for a single DNA molecule, while in our experiments, multiple DSB were present, as manifested by the short DNA fragments. Of course, comparison of the absolute values of these coefficients is not meaningful because of the different DNA molecules and radiations used. Nonetheless, the closeness of the data obtained by the three completely different techniques seems to indicate some fundamental underlying physical mechanisms involved in DNA DSB production. We also attempted a linear fit to the data, but the result was significantly worse, thus excluding the possibility of linear dose-response.

Finally, we attempted a pure quadratic fit to the data, setting b = 0. The results gave c = -0.017 and $R^2 = 0.982$, only slightly poorer than the linear-quadratic fit. Thus, within the present experimental limits, we cannot rule out a pure quadratic fit, even though a linear-quadratic fit is slightly better.

Two other observations are worth mentioning,

which are unique to the AFM technique of DNA studies: we observed enlarged ends, or blunting, associated with many DNA fragments, and kinks were observed with some of the circular DNA molecules. Blunting and kinks are indicated in Figure 1 by arrowheads. At present, we suspect that blunting is probably due to partial unwinding of the DNA double helix at the ends, while kinks may be a result of DNA single strand breaks or inter-/intra-strand cross links. Further experiments will be conducted to verify these observations.

Conclusions

The AFM analysis of DNA following exposure to ionizing radiation provides a direct view of the resultant fragments. Measurement of the lengths of these DNA fragments permits an estimate of the number of DSBs, which appear to be correlated by a linear-quadratic model. Examination of the ends of the broken strands also shows a blunting, which we interpret as a partial unwinding of the DNA double strand. The resolution and sensitivity of AFM imaging supports an important role for this technology in the analysis of the interaction of radiation with DNA.

Acknowledgments

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Discussion with Reviewers

T.M. Seed: The authors suggest that this technique might well represent a greatly improved method, with increased sensitivity in measuring radiation-induced DNA damage. However, the range of doses applied are very high and extend into the pupralethal range (applied whole body exposures) of exposure doses. In terms of exposure doses, these measurements of radiation damage to critical molecules are far from sensitive. Clearly, the more biologically relevant doses are at a much lower range of doses. My question to you is whether or not your AFM imaging procedure is amenable to exposure doses say below 1 Gy or even below 10-20 Gy?

C.K. Hill: Since the current research is being aimed at solving the mechanism of double strand break production at biologically important doses, can the authors comment on the likelihood of being able to detect DSBs at such lower doses using this technique. Presumably this is a question of statistics not resolution?

Authors: In principle, even a single DNA double strand break can be visualized by AFM. Since ionizing radiation interaction with DNA is believed to be stochastic in nature, the only limitation that we face is the scanning time and the number of DNA molecules that need to be examined. Using higher doses, the frequency of strand breaks change, but not necessarily the characteristics.

A. Schaper: What about the influence of single strand breaks (nicking) on the DNA conformation? Is the efficiency of fragmentation influenced by the extend of nicking?

Authors: Nicking changes a supercoiled plasmid into relaxed circular form. In our experiments using AFM, we have observed kinks with the circular DNA molecules, which could be caused in part by single strand

breaks. Chadwick and Leenhouts (1981) have hypothesized that single strand breaks in close proximity can become double strand breaks, which suggests that DSBs are influenced by the nicking. However, we have not experimentally addressed this question in this paper.

A. Schaper: Of course, the AFM can provide statistical information from the analysis of the topographic data. But, complementary results can be obtained by electron microscopy (EM). I am still wondering about why EM should not be applicable to the radiation damage problem of DNA. Could the authors explain why only AFM is suitable for that purpose? They should keep in mind that there are still other limitations of the structural resolution in AFM including drying artifacts, tip-sample convolution, and loading force.
Authors: Electron microscopy has its use in visualizing DNA. However, AFM offers the following advantages: (1) sample preparation procedure is simple and introduces minimal alteration to DNA structure; (2) AFM can be used to image DNA in liquid that may simulate the living environment of DNA; and (3) the loading force can be varied and controlled according to the specimen imaged, for soft biological samples, the loading force is usually maintained at a minimum, which introduces little alteration to sample structure.

Due to these features of AFM, the delicate and easily damaged biological structures can be much better preserved in AFM imaging.

B.D. Michael: Why was freshly cleaved mica used in AFM imaging? Why did rinsing not remove the DNA from the mica?
Authors: Freshly cleaved mica is atomically flat and clean and is a standard substrate on which AFM imaging is performed. Rinsing removes the excess DNA molecules that are not attached to the mica.

B.D. Michael: The percentage of strand breaks does not appear to have been determined, just the average percentage reduction in size.
Authors: Your comments on reduction in DNA size are correct. However, the percentage reduction in size is directly proportional to the number of DSB. Therefore, the functional dependence of size reduction on dose directly reflects the DSB dependence on dose.

J.L. Schwartz: If the dose response is based on the average length of the linear plasmids, would one not expect linear-quadratic dose-response since shorter fragments would require the production of at least two DNA double-strand breaks within the same molecule?
Authors: We agree with you that the quadratic term becomes more significant with the increase of shorter DNA fragments. This can also be seen from examinations of the coefficients of the fitted curve.

J.L. Schwartz: Does the solution that the DNA was irradiated in have any free radical scavenging ability, and could this influence results?
Authors: The solution did not have any radical scavenging ability. We did not test the dependence of DSB production on radical scavenger concentration. However, other researchers (Krisch *et al.*, 1991) have reported that the linear or linear-quadratic dependence of DSB on dose is directly related to scavenger concentration. At low scavenger concentrations, the dose-response is linear-quadratic. As scavenger concentration increases, the dose-response becomes increasingly linear.

D.J. Mueller: One of the great advantages of AFM is that this microscopic technique allows biological systems to be imaged in buffer solution to maintain their native state. The authors dried the DNA strands after their adsorption to the support and afterwards monitored the sample. Air drying of biological specimen leads to surface stresses and can lead to substantial structural changes (i.e., denaturation). Electron radiation influence the stability of DNA which can damage the double stranded structure. To what extent can it be excluded that adsorption and drying of DNA on mica might induce additional breaks of the irradiated DNA?
Authors: We expect that the gentle drying proves to have negligible effects on DNA molecules.

D.J. Mueller: Magnesium is known to affect the structure of DNA. Might alternative buffer conditions show an influence in your experiments?
Authors: We plan to alter the Mg concentration to see its effect on DNA structure in a future experiment.

Additional Reference

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