Laser and Particle Beams (2002), *20*, 299–302. Printed in the USA.

DOI: 10.1017/S0263034602202219

Chromatin structure modification in an excimer laser field

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(RECEIVED 15 November 2001; ACCEPTED 14 January 2002)

Abstract

Chromatin is the complex of deoxyribonucleic acid (DNA) with proteins that exists in eukaryotic cell nuclei. Chromatin was extracted from livers of Wistar rats and subjected to a 248-nm excimer laser radiation, in doses of 0.5–3 MJ/m^2. An UV excimer laser Iofan 1701, with 40-mJ dose/pulse and frequency of 30 Hz was used. The radiolysis of chromatin was analyzed by (1) ^{1}H-NMR spectroscopy, (2) steady-state fluorescence, (3) time-resolved fluorescence, and (4) fluorescence resonance energy transfer (FRET) methods. The laser action on chromatin determines bigger values of the transverse relaxation time (T_2), which indicates less bound water in the chromatin structure, therefore a more injured one. The chromatin intrinsic fluorescence decreases on laser action, proving the destruction of the chromatin protein structure. By the time-resolved fluorescence we established that the relative contribution of the excited state lifetime of bound ethidium bromide to chromatin DNA diminishes with the laser dose. This denotes single- and double-strand breaks produced in DNA structure. By the FRET method, the energy transfer efficiency and the distance between dansyl chloride and acridine orange coupled at chromatin were determined. The distance increases with laser action. The determination of the chromatin structure modification in an excimer laser field can be of real interest in medical applications.

Keywords: Chromatin structure; ^{1}H-NMR spectroscopy; Laser field; Spectrofluorimetric methods

1. INTRODUCTION

Research concerning the laser action on DNA and polynucleotides (Schulte-Frohlinde *et al.*, 1990) or on nucleoproteins (Kovalsky & Budowsky, 1990) have been reported. The study of laser effects on chromatin—the complex of DNA with proteins occurring in nuclei of eukaryotic cells (Kornberg & Lorch, 1999)—is important. This is because laser therapy is used in the treatment of premalignant and malignant skin tumors (Karrer *et al.*, 2001).

The chromatin structure modification in an UV excimer laser field was put in evidence. The doses for 248-nm excimer laser radiation were 0.5–3 MJ/m^2, with 40-mJ dose/pulse and a frequency of 30 Hz.

We performed an analysis and interpretation of (1) ^{1}H-NMR spectroscopy, (2) steady-state fluorescence (the intrinsic chromatin fluorescence, due to fluorescent aminoacids), (3) time-resolved fluorescence of the complexes of chromatin with the fluorescent ligand ethidium bromide (the determination of the fluorescence lifetimes of the complexes and of the relative contributions of bound and free ligand), and (4) fluorescence resonance energy transfer (FRET; the transfer of energy from a donor fluorophore to an acceptor fluorophore) between dansyl chloride and acridine orange, both coupled to chromatin.

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2. MATERIALS AND METHODS

The chromatin was extracted from livers of Wistar rats, according to standard procedures (Kornberg *et al.*, 1989). We used chromatin samples with 2.5×10^{-4} M ($\cong$100 μg/ml) DNA concentration in 10^{-2} M phosphate buffer, pH = 7. An UV excimer laser radiation, with $\lambda = 248$ nm, from an Iofan 1701 laser, with 40 mJ dose/pulse and frequency of 30 Hz was used. The transverse relaxation time (T_2) of chromatin samples was established with a NKr Aroma 78 spectrometer.

The measurements of fluorescence intensity of the chromatin tyrosine (which exists in histones) were performed at 305-nm emission wavelength (λ_{em}) with 280-nm excitation wavelength (λ_{ex}) and for chromatin tryptophan (which exists in nonhistones), at $\lambda_{em} = 345$ nm with $\lambda_{ex} = 290$ nm.

Ethidium bromide (EtBr; Sigma) was used as the DNA intercalating ligand, in a concentration of 6.25×10^{-5} M and its binding was monitored using time-resolved fluorescence measurements (measuring of the lifetime of fluorescence). The fluorescence response function (Thompson & Millar, 2000) is

$$I(t) = A + B_1 \exp(-t/\tau_1) + B_2 \exp(-t/\tau_2), \quad (1)$$

where $I(t)$ is the time dependent fluorescence intensity, A, B_1, and B_2 are constants, and τ_1, τ_2 are the lifetimes for bound and free states of the ligand.

In the FRET method, a donor fluorophore is excited by incident light and if an acceptor is in close proximity, the excited state energy from the donor can be transferred (Selvin, 2000). This can be realized if the emission spectrum of the donor is perfectly superimposed on the excitation spectrum of the acceptor (Radu *et al.*, 1997). Double fluorescent labeling of chromatin was performed with dansyl chloride (Sigma; $\lambda_{ex} = 323$ nm, $\lambda_{em} = 505$ nm) and acridine orange (Gurr; $\lambda_{ex} = 505$ nm, $\lambda_{em} = 530$ nm). The dansyl chloride reacts, under mild alkaline conditions, with α- and ϵ-amino groups of the proteins, the cysteine sulphydryl group, the histidine imidazole group, and the tyrosine phenolic group, while the acridine orange is intercalated between DNA base pairs.

The Förster energy transfer efficiency is defined as

$$E_f = (I_A^D/I_A - 1)\cdot\epsilon_A/\epsilon_D, \quad (2)$$

where I_A and I_A^D are the fluorescence intensities of the acceptor in the absence and in the presence of the donor, respectively; ϵ_A and ϵ_D are the molar extinction coefficients of the ligands at excitation wavelength.

The energy transfer efficiency of this process depends on the inverse sixth distance, r, between donor and acceptor, by the expression:

$$E_f = r^{-6}/(r^{-6} + R_0^{-6}), \quad (3)$$

where R_0 represents the Förster critical distance at which 50% of the excitation energy is transferred to the acceptor. If the molar absorption coefficient is in units of $M^{-1}cm^{-1}$, the wavelength in centimeters, and J, the normalized spectral overlap integral, is in units of $M^{-1}cm^3$, the expression of R_0^6 is

$$R_0^6 = 8.79 \times 10^{-25}(n^{-4}Q_d k^2 J)\ \text{cm}^6 \quad (4)$$

where n is the refractive index of the medium, Q_d is the fluorescence quantum yield of donor in absence of the acceptor, and k^2 is the orientation factor for dipole–dipole interaction. For this pair of fluorochromes, $R_0 = 29.72$ Å.

An Aminco Bowman SPE 500 fluorimeter and a time-resolved fluorimeter FL 900 CD (Edinburgh Analytical Instruments) were used.

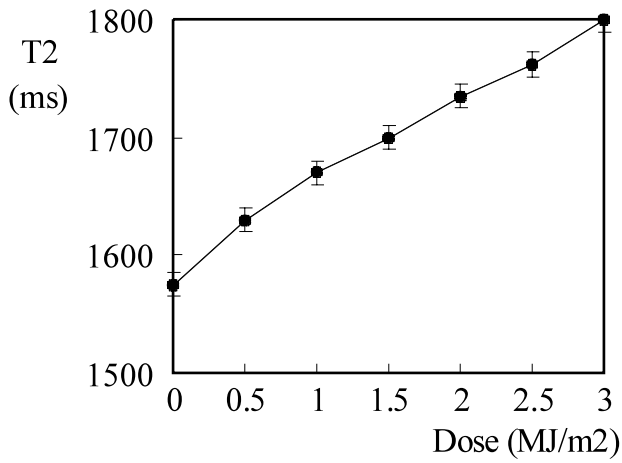

Fig. 1. The chromatin transverse relaxation time versus UV laser dose.

3. RESULTS AND DISCUSSIONS

The dependence of the transverse relaxation time (T_2), obtained by NMR spectroscopy, on excimer laser dose is represented in Figure 1. The increase of T_2 values on laser action indicates less bound water in chromatin, therefore a more injured chromatin structure.

The variations of the chromatin intrinsic (tyrosine and tryptophan) fluorescence intensities on laser dose are indicated in Figures 2 and 3. The modification of the intrinsic chromatin fluorescence indicates a destruction of chromatin protein structure due to laser action. As the protein structure is damaged, the fluorescence intensity of its fluorescent amino acids is reduced.

The time-resolved fluorescence measurements furnished us the lifetimes of the excited states of free (2 ns) and bound (24 ns) EtBr to chromatin. The dependence of the relative

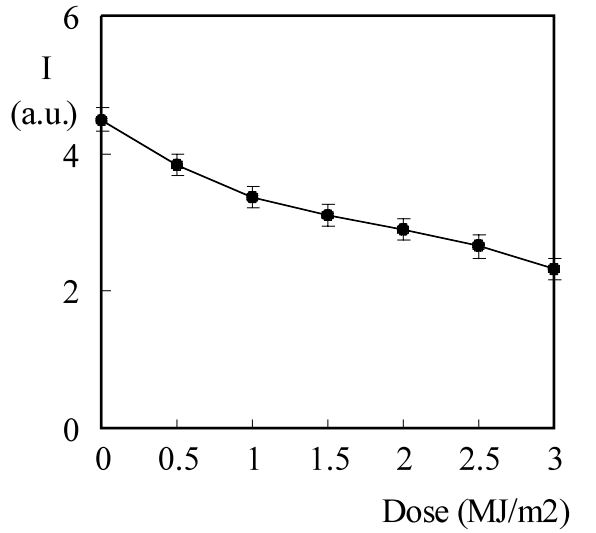

Fig. 2. The chromatin intrinsic relative fluorescence intensities, corresponding to tyrosine, versus UV laser dose.

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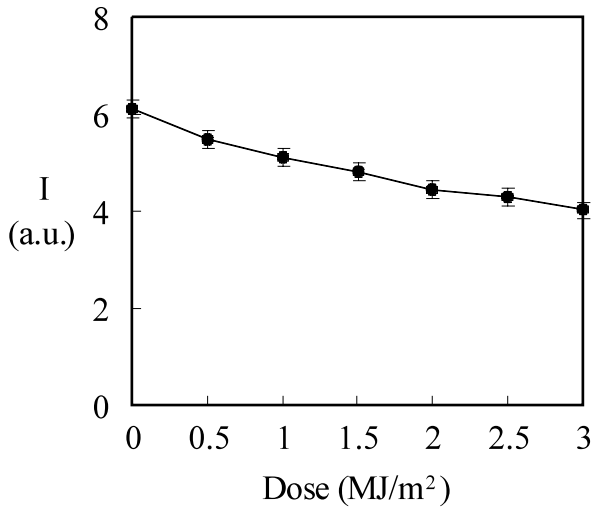

Fig. 3. The chromatin intrinsic relative fluorescence intensities, corresponding to tryptophan, versus UV laser dose.

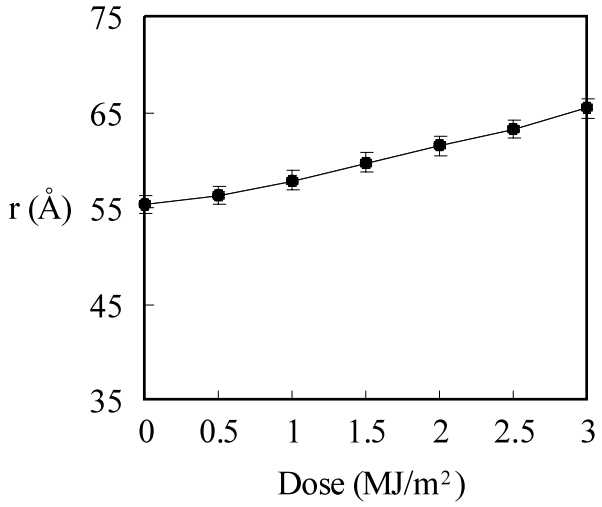

Fig. 5. The average distance donor-accepted between dansyl chloride and acridine orange coupled at chromatin versus UV laser dose.

contribution, f (%), of excited state lifetime of bound EtBr to chromatin on UV excimer laser dose is presented in Figure 4. Since in a free DNA, one EtBr molecule gets intercalated between 2 base pairs (4 nucleotides), all EtBr molecules are bound to a free DNA, when the concentration of DNA is 2.5×10^{-4} M(P) and that of EtBr is 6.25×10^{-5} M. In the case of EtBr binding to chromatin, in the same conditions of concentrations, only a part of the ligand is bound to DNA. This is due to the masking of DNA binding sites by chromatin proteins.

The diminution with laser dose of the relative contribution of the excited state lifetimes of bound ligand constitutes evidence of the reduction of the chromatin DNA double-stranded structure, due to the single- and double-strand breaks.

By using the FRET method, the energy transfer efficiency and the average distance between dansyl chloride and acridine orange coupled to chromatin were determined. The distance r (Å) between donor chromophore and acceptor chromophore varies linearly on laser dose (Fig. 5). The increase of the average distance between the two ligands, under the action of these radiations, reflects the growth of the distance between chromatin proteins and DNA, which suggests a loosening of the chromatin structure.

By using these methods, it was possible to separately follow the radiation effects on chromatin DNA, on chromatin proteins, and on the interaction between DNA and chromatin proteins.

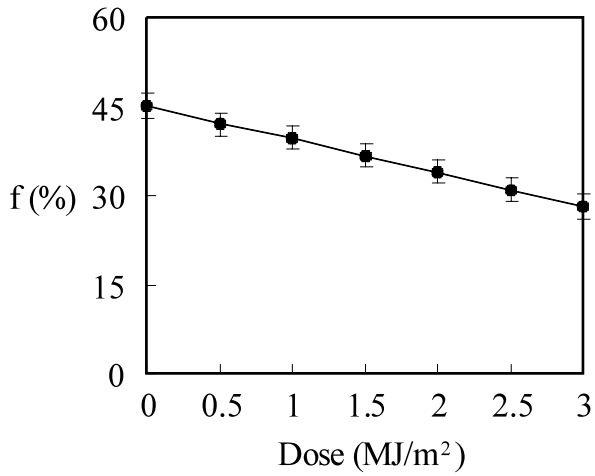

Fig. 4. The relative contribution of the excited state lifetime of bound ligand for chromatin–ethidium bromide complexes versus UV laser dose.

4. CONCLUSIONS

The bigger values of the transverse relaxation time (T_2), indicate less bound water in chromatin, therefore a more injured structure. The chromatin intrinsic fluorescence decreases on laser action, proving the destruction of chromatin protein structure. Using the time-resolved fluorescence we established that the relative contribution of the excited state lifetime of bound ethidium bromide to chromatin deoxyribonucleic acid diminishes with the laser dose. This denotes single- and double-strand breaks produced in the DNA structure. Using the FRET method, we established that the mean distance between chromatin proteins and DNA increases on laser action. The establishment of chromatin structure in a laser field can be important for the understanding of laser action at the biological molecular level in clinical applications.

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