

SHORT COMMUNICATION

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He-Ne laser irradiation protects B-lymphoblasts from UVA-induced DNA damage

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Abstract The effect of He-Ne laser (632.8 nm) pre-irradiation on UVA (343 nm)-induced DNA damage in the human B-lymphoblast cell line NC37 was investigated using the comet assay. He-Ne laser pre-irradiation was observed to result in a dose-dependent decrease in UVA-induced DNA damage. This effect was also found to be dependent on the incubation period between He-Ne laser pre-irradiation and the UVA exposure. Whereas the control cells with a higher DNA damage point to an initial ability of faster repair, both the control and the He-Ne laser pre-irradiated cells subsequently show the same rate of DNA repair. The results suggest that He-Ne laser irradiation protect the cells from UVA-induced DNA damage primarily through an influence on processes that prevent an initial DNA damage.

Introduction

He-Ne laser irradiation (632.8 nm) of cells has been reported to result in a variety of effects on cell structure and function, such as remodelling of the cytoskeletal network [1], formation of giant mitochondria [2], enhanced protein and DNA synthesis [3, 4, 5] and ATP (adenosine triphosphate) generation [6]. Pre-exposure to He-Ne laser irradiation has been observed to modulate the damaging effects of ionising radiations and to lead to a decrease in x-ray sensitivity of *E. coli* K-12 [7] as well as in the cytotoxic effect of gamma-radiation in HeLa cells [8]. It has also been observed to induce protection against UVC radiation damage in wild-type *E. coli* [9]. Although the results of this study suggest singlet oxygen to be involved in the process of induced protection, the

details of the mechanism are not yet quite understood. It was the aim of this study to examine the effect of He-Ne laser pre-irradiation on UVA-induced DNA damage in cultured B-lymphoblast cells.

Materials and methods

Cell culture

Human B-lymphoblast cells (NC 37) were grown in suspension in RPMI 1640 medium (Sigma, Germany) with 10% fetal calf serum at 37°C in a 5% CO₂ atmosphere. The cells were sub-cultured twice weekly in fresh RPMI 1640 medium. For irradiation the cells were centrifuged to remove the medium and resuspended in phosphate-buffered saline (PBS, pH 7.4). The viability of the cells was checked by viewing under a microscope with phase contrast option. The dead cells were easily discriminated visually from live cells because of their darker appearance. Before each experiment, the cell concentration was determined by microscopic examination using a Neubauer haematocytometer and adjusted to approximately 2×10⁶ cells/ml. For light irradiation experiments the cell suspension was placed in a cylindrical cuvette of 0.75 cm diameter and a total volume of 300 µl.

He-Ne laser irradiation

The cell suspension was irradiated by a He-Ne laser (633 nm, ~10 mW) (Uniphase, Calif.) where the beam was expanded with a telescopic beam expander to a diameter of 0.75 cm to ensure uniform irradiation. A 90°-prism was used to direct the beam into the open cuvette from above. Laser power was measured using a Scientech power meter REOS7100 (Boulder, Colo.). With the use of appropriate neutral density filters, the power of the beam was adjusted to ~1.2 mW, resulting in a power density of 27 W/m² at the surface of the liquid in the cuvette. The absorbance of the cell suspension at 633 nm was 0.7. Irradiation was performed in diffused room light at doses ranging from ~0.5 to 2.7 kJ/m² by varying the irradiation time from 20–100 s. During irradiation, the cell suspension was stirred using a 5-mm Teflon-coated magnetic stick moved by a magnetic stirrer under the cuvette to further ensure homogenous irradiation of all cells. Immediately after irradiation the cells were centrifuged and suspended in 1 ml growth medium for re-incubation at 37°C. After variable incubation time periods, the cells were re-centrifuged, washed once with PBS and resuspended in 1 ml PBS. The cell suspension was then used for UVA irradiation. The control sample was processed in the same way except that it did not receive He-Ne laser irradiation.

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UVA irradiation

UVA (343 nm) irradiation was performed using a XeCl excimer laser pumped dye laser (Lambda Physik, Göttingen, Germany), operated at a repetition rate of 20 pulses per second with an energy of ~4.2 mJ per pulse. The energy was measured by a Vector S 200 Energy meter (Scientech, Boulder, Colo.) with a pyroelectric sensor PHD25. The spot size of the beam was 12.5 mm². The energy density of UVA radiation at the surface of the liquid in the cuvette was 6.7 kJ/m². The cell suspension was stirred by a magnetic stirrer to ensure homogenous irradiation of the cell suspension. The absorbance of cell suspension at 343 nm was 0.95. The irradiation dose of 67–135 kJ/m² was achieved by varying the irradiation time from 10–20 s. In all the experiments (except DNA repair experiments) the cells were kept on ice both during and subsequent to UVA irradiation. This was done to retard cellular repair processes and thereby conserve DNA damage induced during irradiation.

Comet assay

After UVA irradiation, the cell suspension was immediately centrifuged for 3 min at 1000 rpm (4°C) and resuspended in PBS. Subsequently cells were embedded in a thin layer of agarose (1:5 with 1% low melting agarose Sigma, USA) on a microscope slide for comet assay. The comet assay was performed as described in [10]. The tail moment of comets (defined as product of comet tail length and the DNA fluorescence intensity in the tail) were measured using computer software Komet 3.0 (Kinetic Imaging, UK) and 100 cells were evaluated for each sample.

Data analysis and statistics

The data analysis was performed using the computer software Sigma plot 3.0. In each experiment the distribution of tail moment in 100 cells/sample was computed as frequency histograms defined as the number of cells vs tail moment. The distribution of tail moments within one cell population can be asymmetric, especially for UVA irradiation at low intensities [11, 12]. Occasionally, such asymmetric distributions are evaluated by two or more Gaussian distributions with different mean values and standard deviations [11, 12]. However, we have shown recently that in order to characterise the state of damage, the fitting of histogram function by the non-symmetric χ^2 -distribution is more applicable than the Gaussian one [10, 13]. These histograms were therefore fitted by χ^2 -function which gives the value of the parameter degree of freedom (n) used to characterise the degree of DNA damage in a given population of cells [12]. The error bar represents standard error of the fitted value. Since the parameter degree of freedom corresponds to tail moment, in the following we express this as χ^2 -tail moment to indicate that this quantity replaces the Gaussian mean of tail moment [13].

Each experiment was performed at least 2 or 3 times to confirm the reproducibility of the data. There was a variation of about 25% in the tail moment of the UVA-exposed cells between the experiments performed with cells cultivated for 3 or 4 days, respectively. This may be due to the fact that in a cell population, the distribution of cells with respect to cell cycle position is expected to vary with the time of cell cultivation. Since the sensitivity of cells towards DNA-damaging agents has been reported to be highly dependent on the cell cycle position [14], a difference in the sensitivity to UVA-induced DNA damage is expected for cells cultivated for different time periods. Therefore, for each experiment, the influence of He-Ne laser irradiation on DNA damage was analysed separately by comparing with the control used in the same experiment.

Results

Figure 1a,b shows the histograms for the distribution of tail moments of 100 cells for cell samples having

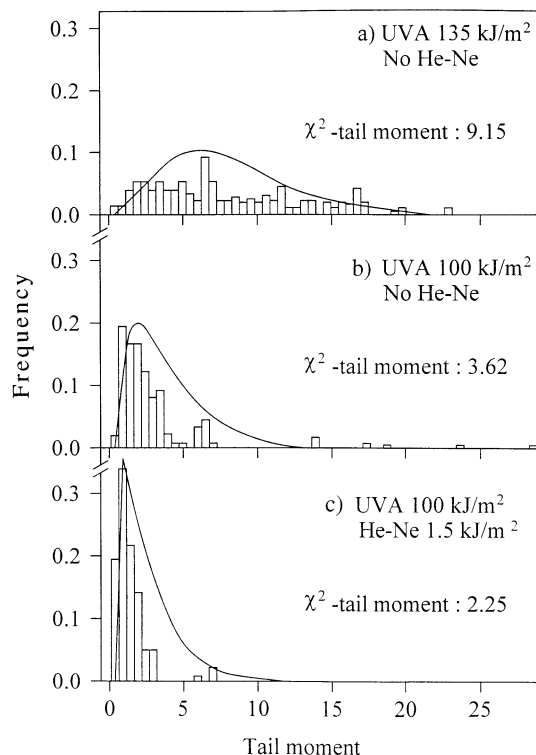


Fig. 1a–c Distribution of tail moments of 100 cells within one population and curve fit with the χ^2 -function. The parameter χ^2 -tail moment characterises the degree of DNA damage. **a** Cells irradiated with UVA radiation at 135 kJ/m²; **b** 100 kJ/m²; **c** cells pre-irradiated with He-Ne laser (1.5 kJ/m²) prior to UVA radiation at 100 kJ/m²

undergone UVA irradiation at 135 kJ/m² (Fig. 1a) and 100 kJ/m² (Fig. 1b). The distribution is asymmetric at lower UVA doses. Figure 1c shows the distribution of tail moments for cell samples pre-irradiated with He-Ne laser at 1 kJ/m² prior to irradiation with UVA at 100 kJ/m². The asymmetry increases due to an increase in the number of cells having lower tail moments. The fitted values for χ^2 -tail moments are indicated in the corresponding figures.

In order to check whether He-Ne laser irradiation by itself could have any effect on the cells, we measured the cell viability as well as the DNA damage after He-Ne laser irradiation. The cell viability measurements show no significant change of the cell survival (Fig. 2a). The data presented in Fig. 2a are the mean and the standard deviations of three independent experiments. For the study of DNA damage, the data of tail moments of 100 cells obtained by the comet assay were plotted as frequency histograms and fitted to χ^2 -distributions. The plot of χ^2 -tail moments as a function of He-Ne laser irradiation dose is shown in Fig. 2b. No difference was observed between the χ^2 -tail moment of He-Ne laser-irradiated cells and control cells, which indicates that He-Ne laser irradiation alone does not result in any DNA damage. It is important to note here that as discussed by Bauer et al. [12], the minimum value that can be taken by the χ^2 -degree of freedom n (used here as χ^2 -tail moment) is 2. Therefore,

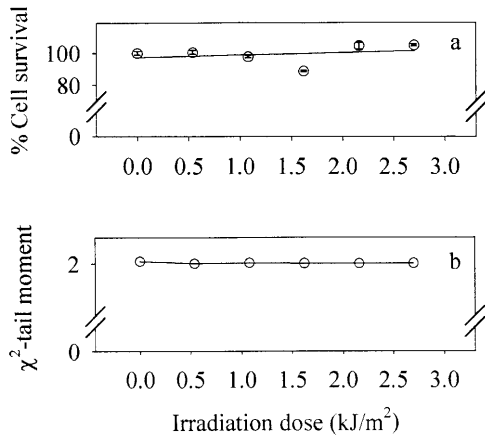


Fig. 2 Percentage of cell survival (*upper panel*) and χ^2 -tail moment (*lower panel*) of lymphoblast cells as a function of He-Ne laser irradiation dose

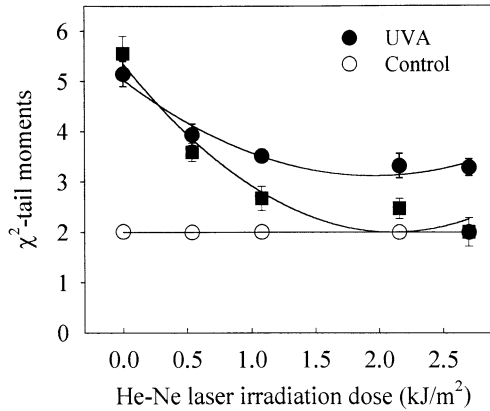


Fig. 3 UVA-induced DNA damage as a function of He-Ne laser pre-irradiation dose with incubation times (37°C) 30 min (●) or 60 min (■) before exposure to 100 kJ/m² of UVA radiation. Controls samples (○) with He-Ne pre-irradiation but without exposure to UVA. Each data point represents the χ^2 -tail moments derived from 100 cells, and the error bars represent standard errors of the fitted value

the value of the χ^2 -tail moment will always be ≥ 2 as seen in Fig. 2b.

Figure 3 shows the χ^2 -tail moments of the control and the UVA-exposed cells (100 kJ/m²) pre-irradiated with He-Ne laser at varying doses for two different periods of incubation between He-Ne laser pre-irradiation and UVA exposure. The incubation periods were 30 min and 60 min, respectively, and the UVA dose was kept fixed at 100 kJ/m² for this experiment. The control cell samples were processed in an identical manner except that they did not receive UVA exposure. For both incubation periods (30 min and 60 min), the pre-exposure of the cells to He-Ne laser irradiation can be seen to result in a significant decrease in the DNA damage as indicated by a decrease in the χ^2 -tail moment of the irradiated cells. The effect of He-Ne laser pre-irradiation can be seen to saturate beyond a dose of >1 kJ/m² (Fig. 3). For a He-Ne laser dose of 1 kJ/m², the difference between the χ^2 -tail

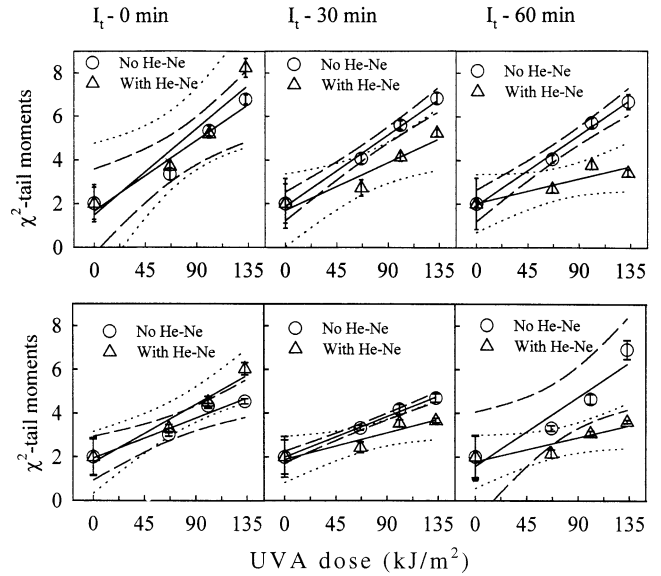


Fig. 4 UVA-induced DNA damage as a function of UVA dose for various incubation periods (I_t) between He-Ne laser (1.0 kJ/m²) irradiation and exposure to UVA in two independent experiments, after 4 days cultivation time (*upper panel*) and after 3 days cultivation time (*lower panel*). The χ^2 -tail moments vs UVA intensity are fitted by linear regression with a value of correlation coefficients of >0.90 for each fit. Each data point represents the χ^2 -tail moments derived from 100 cells and the error bars represent the quality of fit

moment of UVA-exposed cells and control cells was smaller for an incubation period of 60 min compared to that for 30 min. Furthermore, for an incubation period of 30 min the χ^2 -tail moment of UVA-exposed cells remained larger than the control cells even at the highest He-Ne laser pre-irradiation dose applied. With a 60 min incubation period, the χ^2 -tail moment of UVA-exposed cells decreased to the same level as that of the control cells (Fig. 3).

An additional aim of our experiments was to investigate whether the protective effect of He-Ne laser pre-irradiation may be influenced by the UVA dose. The He-Ne laser dose was fixed at 1.0 kJ/m² while the UVA dose varied between 0 and 135 kJ/m². The UVA-dose-dependence was studied for incubation periods of 0, 30 and 60 min. Since there was a variation in the UVA sensitivity of the cells cultivated for different times, experiments on the effect of He-Ne laser pre-irradiation were carried out with the cells cultivated for 4 and 3 days, respectively. He-Ne laser pre-irradiation was observed to lead to a similar protection in both cases, irrespective of the cell cultivation time (Fig. 4a,b). The results show that with increasing UVA dose the DNA damage indicated by the χ^2 -tail moments increases linearly for all experiments (Fig. 4a,b). Therefore the data were fitted by linear regression and a 95% confidence interval was plotted for each curve. With no incubation period between He-Ne laser pre-irradiation and UVA irradiation, no significant effect on DNA damage by He-Ne laser pre-irradiation was observed (Fig. 4a,b). For the 30 min incubation period, He-Ne laser pre-irradiated cells show a lower DNA

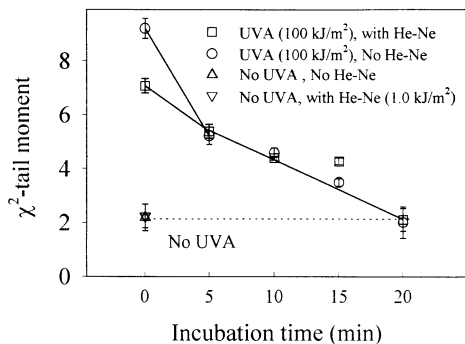


Fig. 5 DNA-repair time kinetics in He-Ne laser pre-irradiated cells (□) and in cells not pre-irradiated (○). Following He-Ne laser pre-irradiation (1.0 kJ/m²) and incubation at 37°C for 60 min, cells were exposed to UVA of a fixed intensity (100 kJ/m²). Subsequently, to allow for DNA repair, cells were incubated at 37°C for various time periods. No UVA, no He-Ne (△), no UVA, but with He-Ne (▽)

damage as compared to the cells not irradiated by He-Ne laser (Fig. 4a,b). There was no overlap between the 95% confidence interval value for the He-Ne laser pre-irradiated cells and non-irradiated cells for UVA doses >100 kJ/m². The protective effect was more pronounced at the 60 min incubation period (Fig. 4a,b).

Figure 5 shows the repair kinetics of UVA-induced DNA damage. In these experiments, both He-Ne laser pre-irradiated and non-irradiated cell samples were incubated at 37°C for 0–20 min subsequent to UVA exposure. As can be seen in Fig. 5, DNA damage decreased with increasing recovery time after UVA irradiation, indicating repair of DNA photolesions. The curve could be fitted by two linear functions indicating repair of DNA damage in a biphasic manner with a fast (0–5 min) and a slow (5–20 min) component (Fig. 5). The presence of a rapid and slow phase of repair is similar to the DNA repair kinetics reported earlier for UVA as well as for gamma-radiation [15, 16]. Immediately after UVA exposure, there is significantly less DNA damage in He-Ne laser pre-irradiated than in non-irradiated cells. The slope of the rapid initial phase from 0–5 min is steeper for cells without He-Ne pre-irradiation, as compared to He-Ne laser pre-irradiated cells. After a recovery time of 5 min, the decrease in DNA damage for both He-Ne pre-irradiated and non-irradiated cells was identical (Fig. 5).

Discussion

Over the past few years, the comet assay or single cell microgel electrophoresis assay has become a valuable tool for sensitive detection of DNA strand breaks and alkali-labile sites [17, 18]. The comet assay allows detection of as little as 0.1 DNA breaks per 10⁹ Dalton [19], requires a relatively small number of cells (1–100000), and results can be obtained in a few hours [17]. Furthermore, since the comet assay also detects apoptotic cells, the measurements performed on a cell-by-cell basis al-

low one to exclude these cells during the measurements, giving an actual estimate of DNA damage and repair [18].

The comet assay technique applied here was used to investigate the influence of He-Ne laser pre-irradiation on UVA-induced DNA damage in B-lymphoblast cells. He-Ne laser irradiation was seen to result in a dose-dependent decrease in UVA-induced DNA damage which was found to saturate beyond a He-Ne laser dose of 1 kJ/m² (Fig. 3). He-Ne laser irradiation alone showed no significant effect on cell survival indicating that it has no cytotoxic effect on cells (Fig. 2a). This also rules out the possibility that the decrease in tail moment may be due to He-Ne laser irradiation-induced formation of DNA crosslinks, (which prevents migration of the tail). Furthermore, because of the short incubation period used in the present study, no red light-induced proliferation of cells [5, 20, 21] was observed. Another possibility is that the He-Ne laser irradiation-induced decrease in tail moment may arise due to an impairment of an excision repair process, which can influence the tail moment by generating DNA strand breaks during repair. However, to avoid the possibility of DNA repair, the cells were kept at 0–4°C throughout the experiments. It is also important to note that for DNA damage induced by wavelengths >330 nm, excision repair does not contribute significantly to the repair of DNA lesions [22, 23]. This is also evident from our studies on DNA repair which show a monotonous decrease of tail moment (Fig. 5) whereas during the excision repair process the tail moment is expected to increase initially due to formation of DNA strand breaks and then decrease [22, 23].

The results presented in Fig. 4 show that the He-Ne laser-induced decrease in DNA damage, depends on the time of incubation of cells between the two procedures of irradiation. A similar dependence has also been observed in our studies on He-Ne laser-induced protection against UVC radiation in *E. coli* [9] and, earlier by Karu et al. in their studies on He-Ne laser-induced protection against gamma-radiation in HeLa cells [8]. However, the processes involved in the development of the protective response are not well understood. It has been argued that the biological response of cells to monochromatic light involves activation of a photoacceptor molecule followed by a cascade of biochemical reactions in the time scale of minutes and hours after irradiation [24]. For example, an increase in intracellular calcium and activation of the ATP-dependent K⁺ ion channel was observed within minutes after irradiation [25, 26], while RNA and protein synthesis peaks at 1 h after irradiation [3, 4]. Therefore, it appears that the protective effect of He-Ne laser involves the induction of biochemical reactions which needs further investigations.

Our study of DNA repair kinetics does not show any significant difference in the rate of DNA repair between He-Ne laser pre-irradiated cells and control cells except for the fact that during the rapid phase, the rate of DNA repair was slower in He-Ne laser pre-irradiation cells compared with the control cells (Fig. 5). This appears to

be due to a difference in the initial (at 0 min incubation) DNA damage level, and suggests that the protective effect of He-Ne laser is manifested through an influence on the processes which inhibit the DNA damage induction.

The mechanism of the He-Ne laser-induced protection appears to be a sort of adaptive response. This follows because He-Ne laser irradiation has been observed to lead to generation of singlet oxygen [27] and the fact that DNA damage induced by UVA is believed to be mediated by reactive oxygen species generated as a consequence of photodynamic activation of endogenous chromophores. Our earlier study on He-Ne laser irradiation-induced protection against UVC exposure in *E. coli* suggested that the effect may be mediated via generation of singlet oxygen [9]. This possibility is also supported by a similar observation of protection of UVC-induced damage in chick embryo cells pre-exposed to short-term magnetic fields [28]. In this study also, the generation of reactive oxygen species by exposure to short term magnetic fields was suggested to play a role in the observed protection. The conjecture that the observed protection is an adaptive response requires that He-Ne laser pre-irradiation by itself should lead to some degree of DNA damage. In the present study no measurable DNA damage was seen to follow He-Ne laser pre-irradiation (Fig. 2b). This may be due to the fact that the sensitivity of the comet assay technique used in our study to detect DNA damage is approximately 300 strand breaks per cell [19]. Therefore, the doses used in our study may result in induction of DNA damage below the detection limit of the comet assay. This possibility is supported by the fact that at higher doses used by other investigators, induction of DNA damage [29] and impairment of cell function has indeed been reported [30, 31].

He-Ne pre-irradiation has also been reported to lead to an increase in the activity of antioxidant enzymes [32, 33]. It is therefore possible that the protection of UVA-induced DNA damage by He-Ne laser irradiation may also be mediated through stimulation of an antioxidant defence system. The relative role of the two possibilities needs further investigations.

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