UVA-INDUCED OXIDATIVE STRESS IN SINGLE CELLS PROBED BY AUTOFLUORESCENCE MODIFICATIONS, CLONING ASSAY, AND COMET ASSAY

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1. ABSTRACT

Cell damage by low-power 365 nm radiation of a 50 W high-pressure mercury microscopy lamp was studied. UVA exposure to CHO cells resulted for radiant exposures >10 kJ/m^2 in significant modifications of NADH-attributed autofluorescence and in inhibition of cell division. Single cell gel electrophoresis (comet assay) revealed UVA-induced single strand DNA breaks. According to these results, UVA excitation radiation in fluorescence microscopy may damage cells. This has to be considered in vital cell microscopy, e.g. in calcium measurements.

Keywords: UVA, NADH, autofluorescence, cloning assay, comet assay, elctrophoresis, oxidative stress, UV microscopy, vital cell microscopy

2. INTRODUCTION

Since the discovery of ultraviolet (UV) radiation by the 24 years old scientist Johann Wilhelm Ritter from Jena in 1801 and the construction of the first "pure" UV lamp ("Wood's lamp"), UV light has become a useful diagnostic and therapeutic tool in medicine and biology. For example, the well established PUVA psoriasis therapy is based on the application of the photosensitive drug **P**soralen and **UVA** light, the Wood lamp is applied in diagnosis of skin disorders including skin bacteria detection, and UVA light is used as excitation radiation in cell fluorescence microscopy[1,2].

On the other hand, UV radiation may induce genetic as well as nongenetic cell damage. UV-related damage to DNA and RNA is assumed to be either a result of direct absorption of UV photons, or of photooxidation processes after excitation of other cellular endogenous chromophores. Nucleic acids have major absorption bands in the spectral range between 200 and 300 nm (maximum around 260 nm). However, solar photons shorter than 290 nm (UVC) are absorbed by stratospheric ozone. UVB radiation (290-320 nm) induces DNA damage via formation of pyrimidine photoproducts and other nucleic acid base photoproducts. UVA (320-400 nm) is well known to induce reactive oxygen species by photodynamic action of certain endogenous chromophores (singlet oxygen, oxygen radicals) causing oxidative stress[3-6]. Major endogenous absorbers of UVA light are flavin coenzymes, the reduced pyridine coenzymes β-nicotinamide adenine dinucleotide (NADH) and β-nicotinamide adenine dinucleotide phosphate (NADPH), porphyrins, and heme-containing enzymes including cytochromes. Membrane proteins absorb UVA photons poorly due to the absence of significant absorption maxima in the UVA spectral region. When exposed to UVA light, intracellular NAD(P)H emits in the blue/green spectral region. Free NAD(P)H fluoresces at ≈460 nm. When bound to proteins, the confirmation changes from a folded to an unfolded form resulting in an increased fluorescence quantum yield and a blue-shifted fluorescence maximum at ≈440 nm. For example, binding of NADH to alcohol dehydrogenase results in 2fold fluorescence increase. Because fluorescent coenzymes act as highly sensitive bioindicators of respiratory chain activity, monitoring of NAD(P)H attributed autofluorescence provides information on light-induced disturbances to metabolic function[2,7-10].

Aim of this paper is the evaluation of cell damage by the UVA radiation which is used as excitation source in fluorescence microscopy. Classical fluorescence excitation is the 365 nm radiation of a high pressure mercury lamp. UVA effects on cellular metabolism were probed by monitoring the cellular redox-state during exposure, by studying the cell growth and reproductive behavior after exposure, as well as by detection of UVA-induced DNA-damage.

DNA damage was detected by single-cell gel electrophoresis (SCGE), also called comet assay. The comet assay allows the sensitive detection of DNA strand breaks, to differentiate between single- and double strand breaks, and to yield information on repair mechanisms[11-13]. The assay is based on migration of DNA molecules, which carry a net negative intrinsic charge, in a weak electric field. When embedded in a porous medium such as an agarose gel the migration distance is nearly

inversely proportional to the logarithm of the molecule length. Therefore, smaller DNA fragments (e.g. induced by UVA exposure) migrate further towards the anode than larger ones. Intact DNA remains in the nucleus. When stained with a fluorescent dye DNA migration can be detected. The DNA pattern has the appearance of a comet with a head and a tail indicating intact DNA and DNA fragments, respectively. The alkaline SCGE allows detection of "alkali labile sites" and single-strand breaks, whereas the neutral assay detects double-strand breakes. The comet assay has so far been applied for chemical-, UVC-, UVB-, and ionizing radiation-induced DNA damage[14-17]. As few as 1 DNA break per 10^{10} dalton can be detected[15].

3. MATERIALS AND METHODS

Cells

Chinese hamster (Cricetulus griseus) ovary cells (CHO-K1, ATCC no. 61) were used in the UVA experiments due to their rapid reproduction time of about 12 hours and their ability to grow in low cell concentration. This allows clonal growth studies on single isolated cells. CHO cells were maintained in GIBCO's minimum essential medium (MEM) supplied with 10% fetal bovine serum. For autofluorescence and clonal growth studies, cells were grown in sterile Rose chambers consisting of two 0.16 mm coverslips as chamber windows, a silicon gasket with a 2 cm opening as spacer, and metal frames.

Autofluorescence Monitoring

Intracellular autofluorescence imaging was performed with an inverted epifluorescence microscope (Zeiss, Germany). Fluorescence excitation and UVA radiation was provided by the 365 nm line of a 50 W high-pressure mercury lamp. UVA power was measured (Newport) in air after the Neofluar 100x phase contrast objective near the sample plane to be 1 mW (power meter: 818-UV, Newport Coop., Irvine, CA). The homogeneity and area of the UVA radiation transmitted through the 100x objective was measured with UV sensitive photochromic detection layers. These transparent layers contain photosensitive spiropizane compounds which undergo reversible heterolytic bond breakages during UV exposure resulting in fluorescent photoproducts with absorption bands in the blue, and lifetimes of several minutes[18]. A diameter of the circular UVA irradiation area of 0.190 mm was detected by confocal fluorescence imaging (λ_{exc} = 488 nm, λ_f > 510 nm). Therefore, the UVA intensity at the sample is about 35 kW/m^2.
Autofluorescence images were recorded in the spectral range of 430-470 nm using a thermoelectrically-cooled, slow-scan CCD camera (Princeton Instruments) and processed with IPLab software (Princeton Instruments). Fluorescence excitation time was chosen to be 1 s. Autofluorescence imaging was performed at 37°C using an air-curtain incubator on cell monolayers which had been growing for 2 days in the Rose chamber.

Cell Survival and Clonal Growth

Trypsinized CHO cells were injected in a very low concentration (about 100 cells/ml) into Rose chambers. After attachment to the bottom coverslip, six cells of each chamber were preselected and marked by scribing a 50 µm circle around the cell on the outside of the chamber window using a diamond objective (Zeiss). Three of the preselected cells were exposed to focused UVA radiation (100x) 6 hours after injection into Rose chambers. The experimental conditions were the same as for autofluorescence studies. The other three unexposed cells served as controls. Cells in the chambers were maintained in an incubator (5% CO_2, 37°C) up to 5 days after exposure. Circled, selected cells were found again by means of low magnification phase contrast microscopy. The marked cells were observed in 24 hours periods for morphology and clonal growth. Clonal growth was considered to be unaffected by UVA exposure when the cell was able to form a clone of >25 cells after 72 hours.

Alkaline Single-Cell Gel Electrophoresis

For SCGE, attached CHO cells were exposed to 0.4% trypsin for 20 s, shaked, and placed in 6 mm cylindrical cuvettes (≈10^6 cells/ml, 300 µl) filled with MEM medium. The cell suspension was stirred during UVA exposure (magnetic stirrer, H+P Labortechnik, München). The cuvette was kept within an ice-water bath during irradiation to minimize repair reactions. UVA exposure was performed with the unfocused 365 nm beam leaving the microscope (additional diaphragm instead of objective). UVA power was measured to be 22 mW. Considering an 1.5 cm^2 area of exposure at the sample, the mean intensity was 150 W/m^2. The time of exposure was chosen to be 0 s, 60 s, 180 s, 300 s, 600 s, and 1800 s.

The UVA-exposed cell suspension was centrifuged (rpm 1000), diluted with PBS (pH = 7.4) to a final concentration of $4.5x10^5$ cells/ml, and mixed in the ratio 1:5 with 1% low melting point agarose at 45°C (solved in PBS, A4018, Sigma, Germany). The cell-agarose suspension was filled in a microchamber, consisting of a slide and 0.34 mm glass spacers, and covered with a coverslip. After cooling, the coverslip was removed. The microchamber was immersed in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% sodium sarcosinate, NaOH to adjust pH = 10, 1% Triton X-100, 10% DMSO) and kept at 4°C for 1 hour. Cells were then placed in a horizontal electrophoresis tank (0.33 M NaOH, 1 mM Na_2EDTA, pH=14) for 15 min. Electrophoresis was performed at a field strength of 0.5 V/cm for 10 min (Bio Rad, Germany). The weak electric field leads to migration of broken DNA from the nucleus toward the anode forming a "tail". Then, microchambers were washed with Tris buffer, pH = 7.5. Finally, cells embedded in agarose were stained with propidium iodide (PI, 2.5 µg/ml) for 30 min and examined using a fluorescence microscope (Axiovert M 135, Zeiss). The 536 nm radiation of a high pressure mercury lamp (broadband filter: 510-560 nm) was used for PI excitation; PI fluorescence (fluorescence maximum: 610 nm) was detected using a LP590 filter. Fluorescence was imaged with an intensified CCD video camera (model: VarioCam, PCO, Computer Optics GmbH, Kelheim) and analyzed with the software Komet, version 3.0 (Kinetic Imaging Ltd., Liverpool). This software analyzes more than 30 parameters of each comet, such as comet length, head size, amount of DNA in head and tail by comparison of fluorescence areas and intensities. We used the parameter tail moment, defined as the product of tail length and the amount of DNA in the tail, to characterize DNA damage. 100 cells for each exposure time were evaluated. Experiments were performed in dark.

4. RESULTS

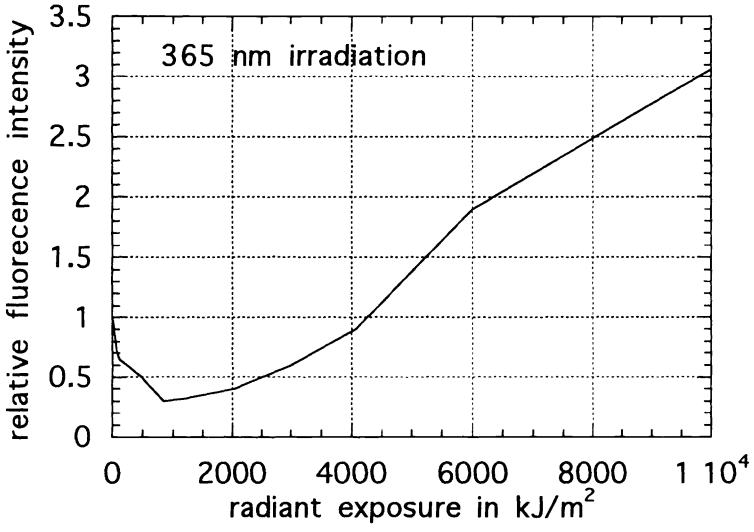
Autofluorescence Monitoring

Cells exhibited a weak fluorescence in the blue spectral region when excited with 365 nm radiation. Fluorescence arose mainly from mitochondria as shown in comparative Rhodamine staining tests. At first, UVA exposure resulted in rapid fluorescence decrease down to ≈30% of the initial value. No morphological damage was observed with phase contrast measurements as well as Trypan blue staining test. The cell autofluorescence reached a minimum after ≈30 s (≈1000 kJ/m^2), followed by a 5fold fluorescence increase within 10 min (≈20 MJ/m^2). Further irradiation results in slow decrease. The increase in fluorescence intensity was accompanied by an autofluorescence relocalization. Interestingly, the whole cytoplasm started to show up with intensities higher than those of mitochondria fluorescence. The nucleus became fluorescent and, finally, the nucleoli turned out to be the most intense intracellular fluorescence sites (Figs. 1 and 2). Cells were no longer able to exclude Trypan blue. They showed membrane blebbing and efflux of highly autofluorescent material in the surrounding PBS medium.

Cloning Efficiency

Single interphase cells were UVA exposed for different irradiation times and incubated for 5 days. A cell was considered to be unaffected by UV exposure if clones consisting of at least 25 cells were produced. The cloning efficiency of control cells (no UV exposure but same experimental conditions, same Rose chambers) was determined to be 94% (188 out of 200 cells produced clones). Significant inhibition of clonal growth was observed after UVA exposure. Fig. 3 shows the mean cell cloning efficiency in dependence on UVA radiant exposure. UVA exposure times as low as 1 s (35 kJ/m^2) resulted already in reduced cloning efficiency. Some of the exposed cells were able to undergo one cell division, but daughter cells were unable to divide. For exposure times >10 s, all exposed cells failed to divide. Morphologically, these cells were visible as giant cells, dead cells with severe membrane damage (membrane blebbing), or shrinked dead cells.

1. UVA-induced modifications of CHO autofluorescence



2. Mean cellular autofluorescence intensity vs. UVA radiant exposure

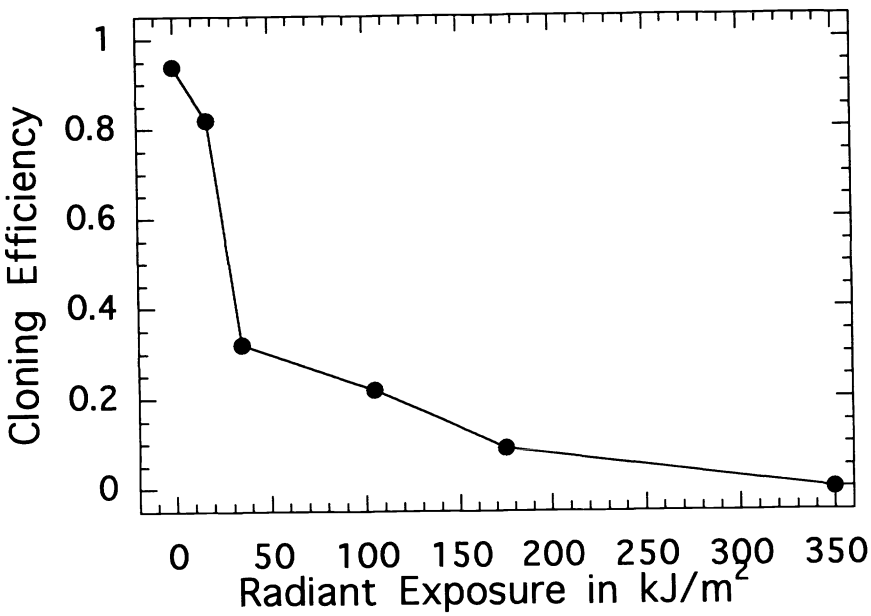


Fig. 3 Mean cloning efficiency after UVA exposure (n =21) vs. radiant exposure

Comet Assay

The DNA migration towards the anode was more pronounced in UVA exposed cells than in control cells which experienced the same procedure but no UVA exposure. Fig. 4 demonstrates a typical "comet" image of a 10 min UVA exposed relatively large cell in comparison with the "comet-free" unexposed cell. The comet tail reached a length of 30 µm whereas the "head" had a dimension of 26 µm (larger size than nucleus diameter prior to electrophoresis). In some cases the tail length can reach 3fold values of the head. More accurate is the use of the parameter tail moment which considers the relative amount of extranuclear DNA (considering area and fluorescence intensity of the tail). The curve in Fig. 5 shows the mean tail moment vs. radiant exposure. A first significant increase occurred already within 1 min exposure (9 kJ/m^2). 30 min UVA exposure (270 kJ/m^2) resulted in 3fold higher tail moments than for the unexposed cells. Curve fitting according the equation for exponential kinetics:

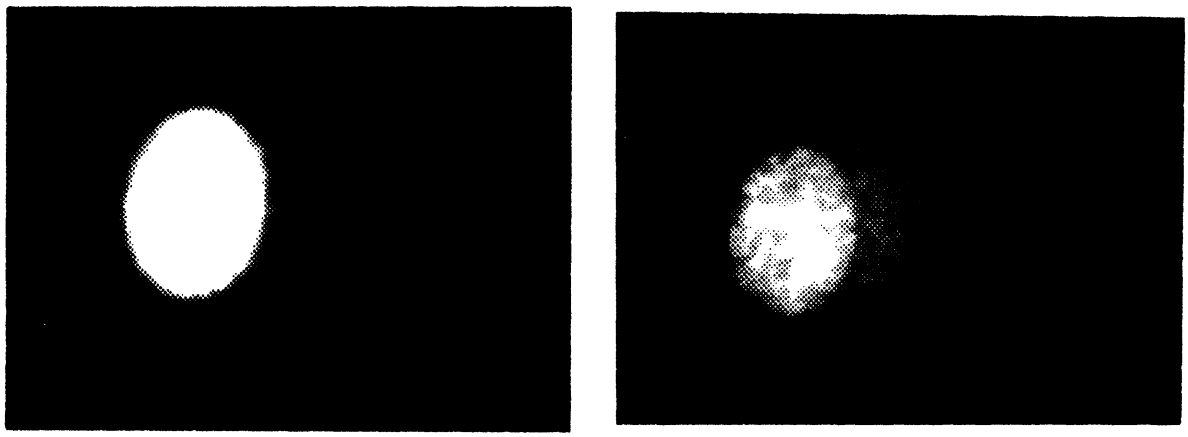
$$y = a + b(1 - \exp(-cx)),$$

(a: tail moment of unexposed cells, a+b: maximum tail moment, c: rate constant)

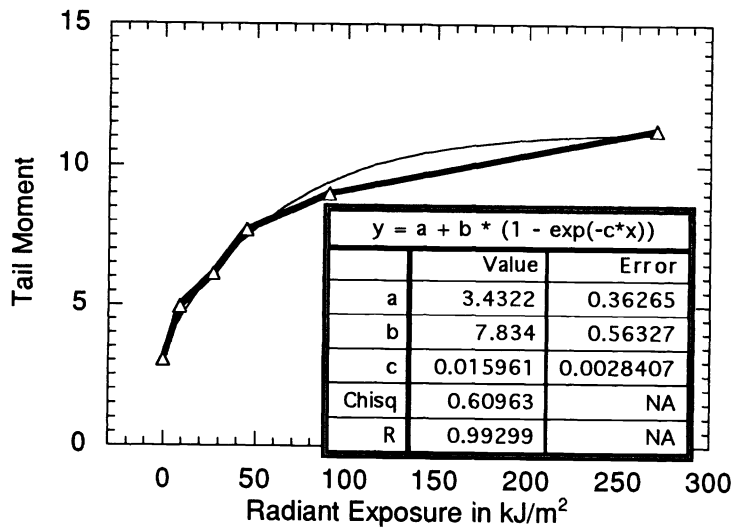
yields parameters a= 3.4±0.4, b= 7.8±0.6, and c= (0.016±0.003)m^2/kJ (R= 0.993). It should be mentioned that within the 100 cells investigated for each exposure time a large variety of the value for the tail moment was observed. This fact is demonstrated in Fig. 6 showing tail moment histograms. A tail moment <4 occurred even in 10% of the 30 min irradiated cells. However, 68% of the UVA-exposed cells in contrast to 4% of the unirradiated cells have tail moments >7.

5. DISCUSSION

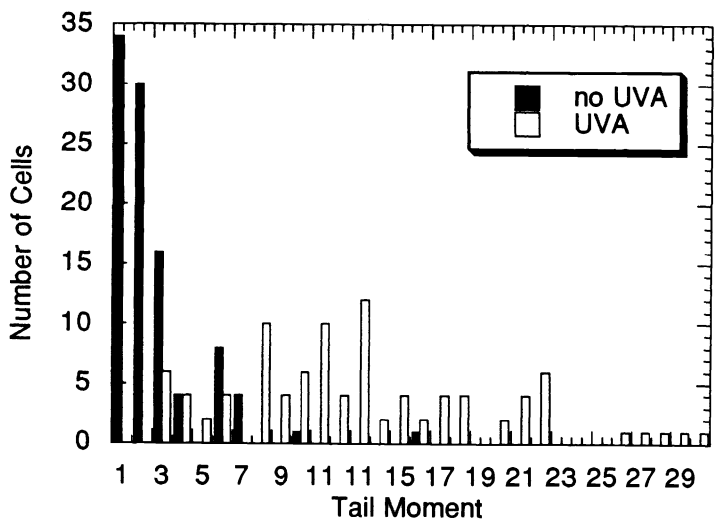
We found that continuous wave low-power UVA radiation, which is used in fluorescence microscopy as excitation radiation, affects cell metabolism. This includes changes in cellular redox state, impaired cell division, and DNA damage. The autofluorescence intensity, indicative for the intracellular redox state, changed during UVA exposure. The initial decrease can be explained with a decrease of NADH concentration due to photooxidation. UVA-induced transformation of NADH to oxidized NAD was found recently (unpublished results). The decrease of autofluorescence would therefore be indicative for a transformation of the cell in a more oxidized state. At this point, cells are still able to exclude Trypan blue. Further UVA



4. Fluorescence images of PI labeled DNA of a single cell embedded in agar after lysis and electrophoresis. Left: non-UVA exposed cell; right: "comet image" of a UVA-exposed cell (270 kJ/m^2)



5. Mean tail moment vs. UVA radiant exposure (alkaline comet assay)



6. Tail moment histogram of UVA-exposed cells (270 kJ/m^2) and non-exposed cells

exposure (radiant exposures >1000 kJ/m^2) resulted in strong fluorescence increase and fluorescence relocalization. This correlated with destructive effects including severe damage to the outer membrane. An explanation for the onset of strong extramitochondrial fluorescence is the destruction of mitochondrial membrane and efflux of NAD(P)H in the cytoplasm. Mitochondria damage could occur as a result of oxidative stress (UVA-induced formation of reactive oxygen species). The diffusion of photosensitizing mitochondrial chromophores including NAD(P)H) in the cytoplasm and UVA exposure leads to damage to nuclear membranes and outer cell membranes. This induces NAD(P)H fluorescence in the nucleus and extracellular medium. Binding of NAD(P)H to the protein-rich nucleoli enhances the NAD(P)H fluorescence quantum yield and leads to intense nucleoli fluorescence. Besides NAD(P)H binding to extramitochondrial proteins the increase of cellular autofluorescence may be also a result of cell transformation in a reduced state as well as enhanced NAD(P)H biosynthesis.
A very sensitive indicator for cell damage is the cloning assay. Interestingly, we found that UVA radiant exposures as low as 35 kJ/m^2 are efficient to inhibit clonal growth. At these radiant exposures, no significant autofluorescence modifications nor morphological changes were detected. Therefore, the cloning assay seems to be more sensitive than on-line autofluorescence microscopy, transmission microscopy, and normally used viability tests such as the Trypan blue exclusion test.
The low power UVA radiation is able to induce DNA strand breakes. We observed first damage in some cells for radiant exposures as low as ≈10 kJ/m^2. Severe damage was detected for >90% of the cells after 270 kJ/m^2 UVA exposure. Gedik et al. [15] found in the case of UVC exposure single strand breakes at radiant exposures as low as 0.5 J/m^2.
UVA-induced inhibition of mitosis of NIH fibroblasts at 360 nm was found for radiant exposures of about 10 kJ/m^2 by Lubart et al.[19]. This value corresponds with our findings obtained for a different cell type.
Our results demonstrate clearly that the fluorescence excitation radiation used in cell fluorescence microscopy is capable to damage the sample. This has to be considered in vital cell microscopy. A wide-spread application field of vital cell fluorescence microscopy is the measurement of intracellular calcium where fluorescent calcium indicators are excited with UVA light. New directions in vital cell microscopy are the excitation of UV transitions via two-photon excitation microscopy. This novel microscopy technique employs highly focused cw or pulsed near infrared (NIR) laser beams[20,21]. Out-of-focus cell regions experience only the relatively harmless low-intensity NIR radiation, whereas excitation of UV transitions by simultaneous absorption of two NIR photons occur in the focal region (high intensity). This should result in reduced photobleaching and photodamage. However, up to now no systematic studies on cell damage induced by pulsed NIR microbeams exist.

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