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THE EFFECT OF UV IRRADIATION ON CHANGES IN CYTOSKELETON AND VIABILITY OF MOUSE FIBROBLASTS L929 CELL LINE

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A b s t r a c t

The effect of long wavelength UV radiation on the survival of L929 mouse fibroblasts and on the integrity of the two main cytoskeletal structures, i.e., microtubules and microfilaments, was studied in relation to the duration of reparation periods. A lamp that emitted radiation at 356 nm was used as a source of UVA. All cells were irradiated with an equal dose of UVA, subsequently reincubated and collected at regular intervals for further processing. Cytoskeleton morphology observations, mitotic index assessment and cell viability tests were performed by fluorescence microscopy. The proliferating activity of cells after irradiation was measured by spectrophotometry using the NRU-assay. Marked damage to the integrity of microtubules was observed immediately after exposure, but no effect on cell morphology was detected. After short reparation periods from 30 min to 3 h, there was an increase in the number of apoptotic cells with typical features (plasma membrane blebbing, DNA fragmentation). The surviving cells were characterised by other changes in morphology, i.e., shrinking of normal cells and a gear-wheel shape of giant multinuclear cells. As the reparation periods prolonged, the proportion of living cells increased and that of apoptotic cells decreased; this was a result of proliferating activity of viable cells and detachment of dead cells from the substrate. The surviving cells showed some abnormalities of the cytoskeletal structures, manifested as atypical arrangements of individual fibres and their asymmetric distribution in relation to the nucleus. After 48 h of reparation, the cell population showed normal parameters of both viability and the morphology of cytoskeletal components.

K e y w o r d s

ultraviolet radiation, cytoskeleton, mouse fibroblasts, L929 cell line, microtubules, microfilaments, mitotic index, viability, proliferating activity

A b b r e v i a t i o n s u s e d

BSA, bovine serum albumin; DAPI, 4,6-diamidino-2-phenylindol; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum, FITC, fluorescein-isothiocyanate, Hsp, heat shock proteins; NRU, Neutral Red uptake; NHEK, normal human epidermal keratinocytes; PBS, phosphate buffer saline; TNF-α, tumour necrosis factor α; UV, ultraviolet; UVA, ultraviolet waveband A; UVB, ultraviolet waveband B; UVR, ultraviolet radiation

INTRODUCTION

Exposure of organisms to ultraviolet radiation (UV) and its resulting effects on the cell structure and functions are currently ones of the most topical fields of

research. A large number of data on clinical sequelae of UV irradiation, such as erythema, changes in pigmentation, carcinogenesis, etc., has recently been extended by the detailed information on reactions of the cell at the molecular level, e.g., Hsp or TNF-α synthesis, activation of early-response genes or protein kinase induction *(7, 3, 2)*.

From the viewpoint of biochemistry, nucleic acids and lipid molecules are the most important sites of direct impact in the cell. In addition to this direct effect of UVR on cell structures, the consequences of which result especially in the formation of pyrimidine dimmers, there are indirect effects of UVR, when the irradiation causes damage to cell structures by oxidative stress (*5*). At the molecular level, chromophores without DNA absorb photons; this absorption results in the production of reactive oxygen species and energy transfer to the target molecules, i.e., DNA, proteins and lipids (*8 and others*). The reactions of oxygen species play an important role in causing DNA lesions as well as in changes in metabolism related to cell damage and induction of apoptosis.

So far little attention has been given to the effect of UVR on the cytoskeletal structures of eukaryotic cells. It is assumed that the reactive oxygen species-mediated oxidative modification of membrane lipids and cytoskeletal proteins is the principal mechanism of cytoskeleton damage in consequence of UVR (*4*). A direct influence on cytoskeletal components was demonstrated in cells *in vitro* after exposure to UVA or UVB radiation (*9, 11, 5*). As a result of UV irradiation, changes in the structure of microtubules due to damage to precursor molecules have been reported in human skin fibroblasts (*10)* and failure or defects of tubulin dimmer polymerisation have been described in an *in vitro* cell-free system (*12*). Similar defects in actin subunit polymerisation and changes in microfilament network organisation have also been observed in human fibroblasts after exposure to UVA (*1*).

The aim of this study was an assessment of the effect of long wavelength UVR on microtubule and microfilament organisation in mouse L929 cells. In addition to the immediate reactions of the cytoskeleton to UVR exposure, we focused on the reparation capability of the cell population in relation to the time period after irradiation. The parameters of viability and proliferating activity of the cell population were studied by assessment of the mitotic index, determination of a live/dead cell proportion and measurement of growth curves using the NRU-assay.

MATERIAL AND METHODS

Cell cultures

For experiments, the mouse fibroblast cell line L929 was used. Cells were grown in DMEM medium supplemented with 10% FCS (PAA Laboratories), 2mM glutamine (PAA Laboratories), 100 $IU.ml^{-1}$ penicillin (PAA Laboratories)

and 100 μg.ml^{-1} streptomycin (PAA Laboratories) at 37°C in an atmosphere of 95 % air : 5% CO_2. They were subcultivated three times weekly.

A cell suspension at a concentration of 10^5 cells per ml was inoculated onto glass coverslips for the assessment of cytoskeletal structures, mitotic index and cell viability. For the NRU-assay, cells in suspension at a concentration of 10^4 cells per ml were seeded into 96-well microtitre plates in a volume of 200 μl per well. The cells were allowed to grow under standard conditions for 24 h.

UV irradiation and reparation conditions

A Balzers lamp emitting UVA radiation at a wavelength of 356 nm and an intensity of 8 W was used. Plastic Petri dishes containing the cells growing on coverslips or microtitre plates were placed, without covers, in a sterile hood and irradiated at a distance of 5 cm for 30 min. The cells were fixed immediately after irradiation or reincubated under standard conditions for the following periods: 30 min, 1 h, 3 h, 24 h and 48 h.

Visualisation of cytoskeletal structures

The cells were rinsed with PBS (pH, 7.4) and fixed with 3% *para*-formaldehyde (Sigma) in PBS for 10 min at room temperature. After washing in the same buffer, cells were permeabilised with 0.2% Triton TX-100 (Sigma) in PBS for 1 min at room temperature. The coverslips were subsequently washed with PBS and incubated for 10 min with 0.2 % BSA to block the non-specific binding of secondary antibodies.

Microtubules were stained by indirect immunofluorescence with the use of mouse monoclonal anti-α–tubulin antibody TU-01 (Exbio) for 1 h at 37°C. Coverslips were washed three times with PBS and treated with swine anti-mouse antibody conjugated to FITC (Sevac) for 45 min at 37°C. Microfilaments were labelled directly with phalloidin conjugated to rhodamine (Molecular Probes) for 1 h at 37°C; phalloidin bound specifically to F-actin.

Finally, the coverslips were washed with PBS and mounted onto glass slides, and fluorescence was observed in a Leitz Laborux epifluorescence microscope. Photographs were recorded on Fujicolor Super G 400 negative film.

Staining of nuclei and assessment of the mitotic index

Following the visualisation of cytoskeletal structures, the cells were mounted, as described above, onto glass slides in the Vectashield mounting medium (Vector) with an addition of DAPI fluorescent dye (Sigma) specifically staining DNA molecules. The mitotic index was determined as the number of mitoses per 100 cells.

Cell viability assay

The proportions of living and dead cells in a population were assessed with the use of a LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes). Two fluorescent probes, calcein AM and ethidium homodimer-1, were simultaneously added to the cells growing on coverslips and incubated for 30 min at 37°C. Calcein AM is a fluorogenic esterase substrate and the resultant green fluorescence of cells indicates both esterase activity and intact membranes. Ethidium homodimer-1 is a red fluorescent dye with affinity to DNA and stains only dead cells after passing through damaged membranes. On each coverslip, 200 cells were examined with the Leitz Laborlux epifluorescence microscope.

Measurement of proliferating activity

The proliferating activity of cell populations after UVA irradiation was determined by the NRU-assay based on the ability of living cells to take up Neutral Red dye from the medium and retain it in their lysosomes. The cells growing in 96-well microtitre plates were assessed immediately after irradiation or after reincubation for the periods defined above.

For this assay, the culture medium was removed and replaced with fresh DMEM containing 2% Neutral Red (Sigma). Subsequently, the cells were incubated for 3 h under the standard conditions, fixed and absorbance was measured at 570 nm using a Spectra Shell (SLT) microplate reader.

RESULTS

Microtubules

The control cells had a characteristic shape with a network of evenly distributed cytoplasmic microtubules *(Fig. 1*). Immediately after exposure, the irradiated cells showed marked disorders of the microtubular cytoskeleton, i.e., fragmentation of individual fibres (*Fig. 2*) and "thinning" of the microtubular network, particularly at peripheral regions of the cell (*Fig. 3*). Neither of these had any affect on cell morphology. After the first two reparation periods (30 and 60 min), the cells began to show impaired adherence; normal cells became rounded (*Fig. 4*) or multinuclear giant cells took a shape reminiscent of a gear wheel. Microtubules were accumulated in the perinuclear region (*Fig. 4*). However, in the cells that retained their original shapes, defects of microtubular integrity manifested as fragmentation of the fibres were usually found (*Fig. 5*). An increasing number of cells with signs of induced apoptosis, i.e., plasma

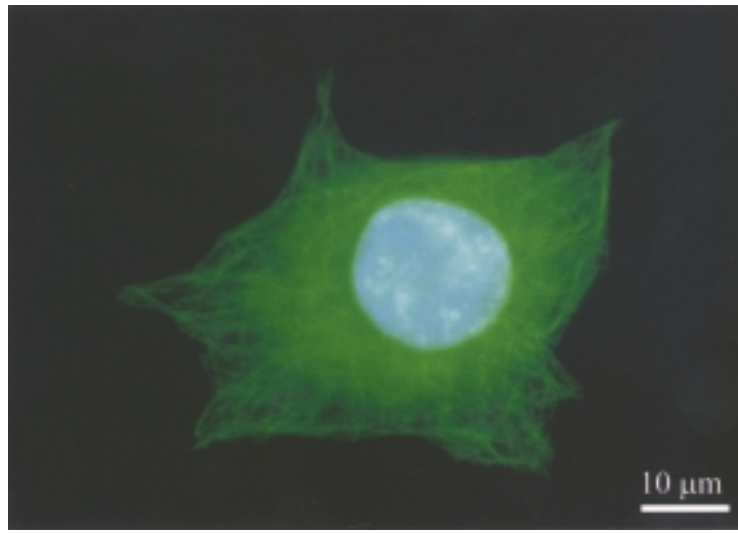

Fig.1
Control L929 cell:
double staining for tubulin (green) and DNA (blue)

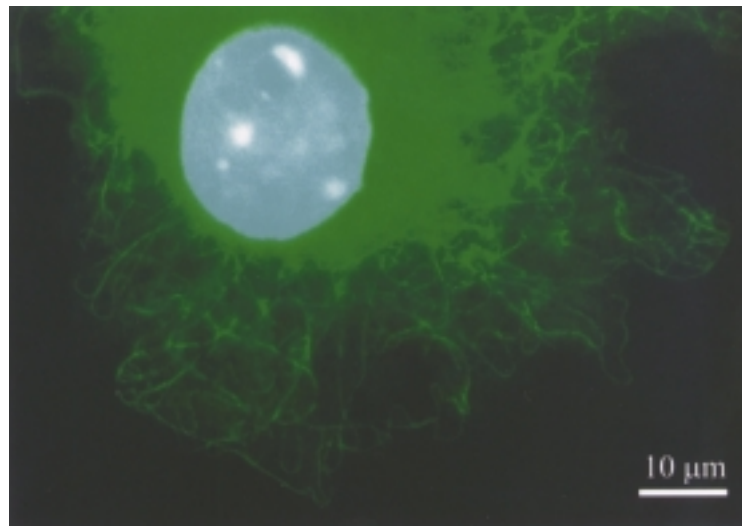

Fig.2
L929 cell immediately after irradiation:
double staining for tubulin (green) and DNA (blue)

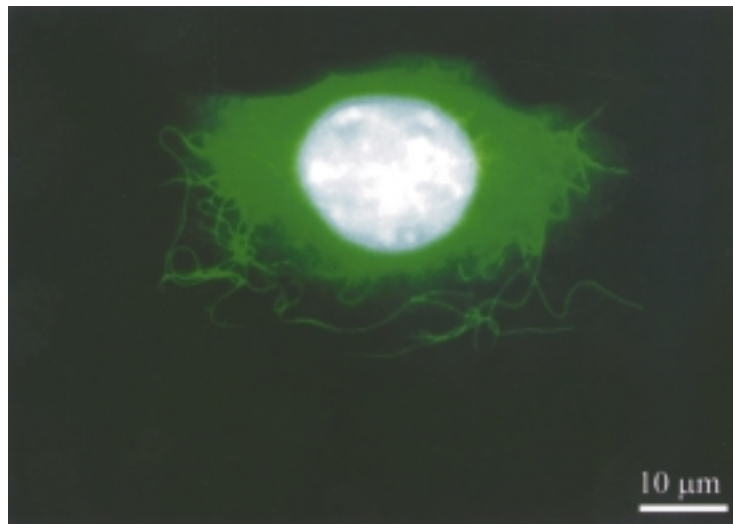

Fig.3
L929 cell immediately after irradiation:
double staining for tubulin (green) and DNA (blue)

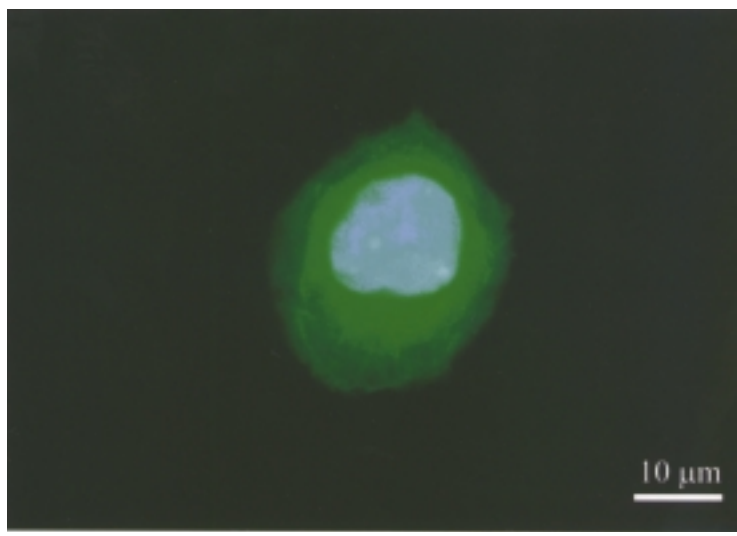

Fig.4
L929 cell at 30 min after irradiation:
double staining for tubulin (green) and DNA (blue)

membrane blebbing and nuclear fragmentation, was also observed. After 3 h of reparation, cell adherence was gradually restored, even though damage to microtubule integrity was still detectable (*Figs. 6, 7*). At 24 h after irradiation, cell adherence was completely restored, but the cells had atypical shapes characterised by many anomalous protrusions (*Fig. 8*). At 48 h, these morphological abnormalities disappeared and the cell population showed normal attributes.

Microfilaments

Morphology of the microfilament network in the control, polygonally-shaped cells is shown in *Fig. 9.* In these cells, thickened longitudinal bundles of actin filaments, termed "stress fibres", were also present. Immediately after exposure, the irradiated cells, began to display impaired adherence, became rounded and the number of stress fibres increased (*Fig.10*). After 30 min of reparation, in addition to these features, some structural abnormalities of individual microfilaments, reminiscent of "knots", occurred in many cells (*Fig.11*). After longer reparation periods (1 h and 3 h), the surviving cells, as they restored their adherence, formed atypical, short and very thin protrusions along the circumference; at the same time, the microfilament network became markedly thinner (*Fig.12*). The morphology of cells at 24 h after irradiation was similar to that described in the cells examined for the microtubular cytoskeleton, i.e., the cells showed atypical shapes and anomalous protrusions. At 48 h after UVR exposure, the appearance of the cell population corresponded to that of the control cells.

Mitotic index

No significant difference in mitotic index values was found between the cell population immediately after irradiation and the control cells (*Fig. 13*). A distinct decrease in mitotic activity was detected in the irradiated cell population after the 30-minute reparation period and this effect also persisted during 1-hour and 3-hour periods. Mitotic activity was fully restored after 24 and 48 h of reparation.

Cell viability

Our results showed that, immediately after exposure to UVR, the proportion of dead cells in the population increased rapidly (*Fig. 14*). However, after 30 min of reparation, a decrease in the proportion of dead cells was noted. This fact was probably caused by release of dead cells from coverslips into the medium. The proportion of dead cells continued to decrease during the following 3 h and, at 24 and 48 h of reparation, it achieved a value similar to that shown by the control cells.

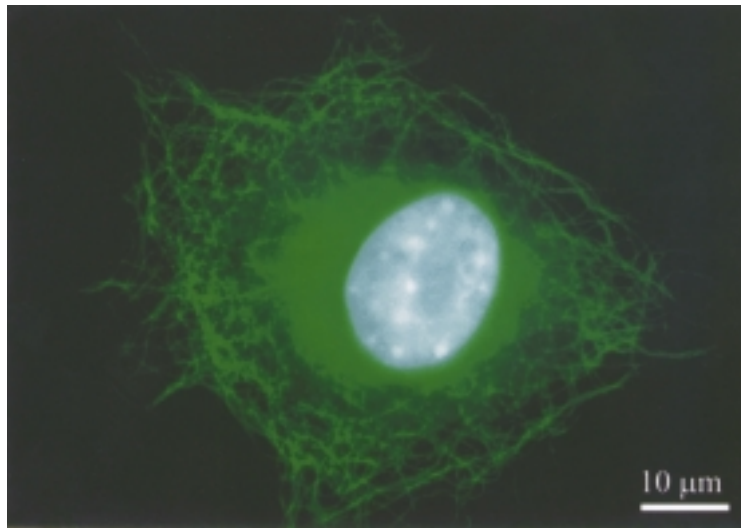

Fig.5
L929 cell at 60 min after irradiation:
double staining for tubulin (green) and DNA (blue)

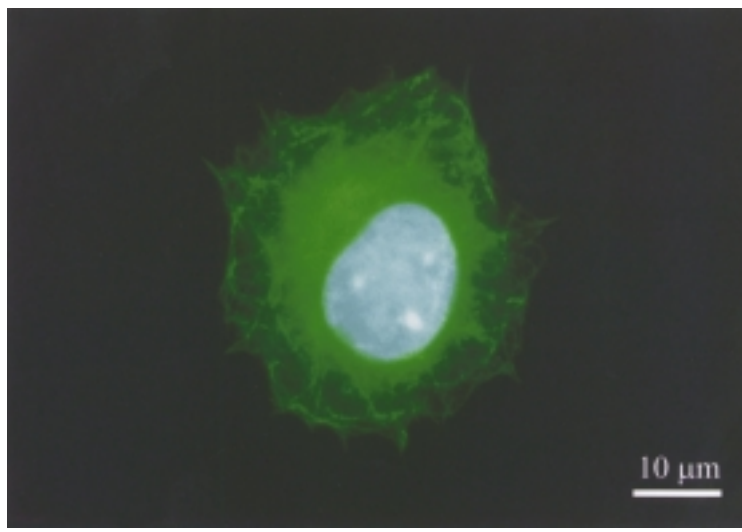

Fig.6
L929 cell at 3 h after irradiation:
double staining for tubulin (green) and DNA (blue)

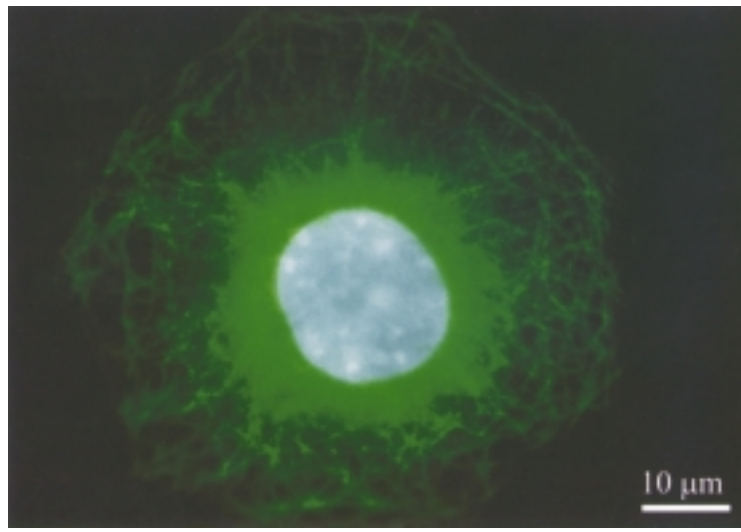

Fig.7
L929 cell at 3 h after irradiation:
double staining for tubulin (green) and DNA (blue)

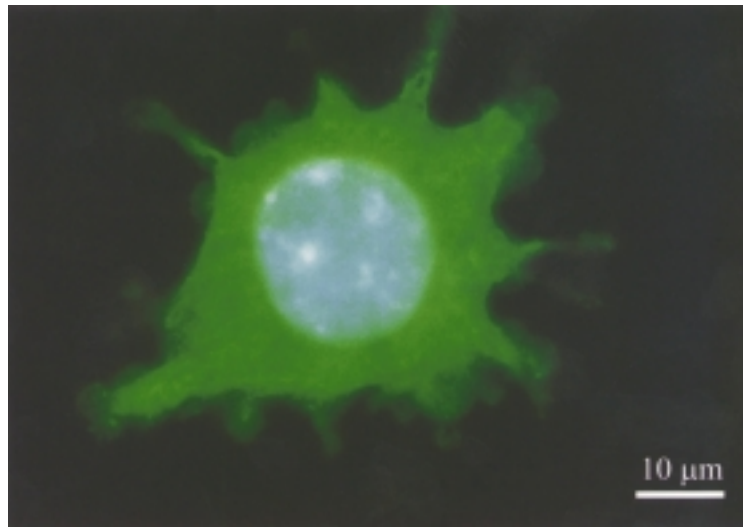

Fig.8
L929 cell at 24 h after irradiation:
double staining for tubulin (green) and DNA (blue)

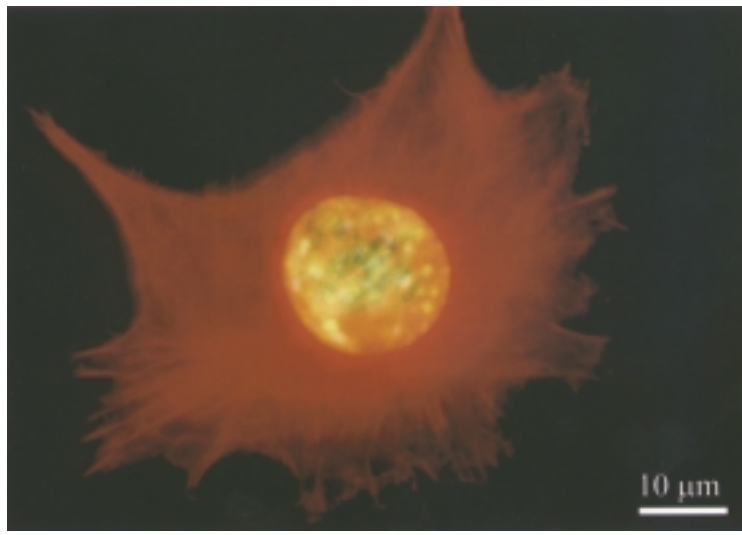

Fig.9
Control L929 cell:
double staining for actin (red) and DNA (blue)

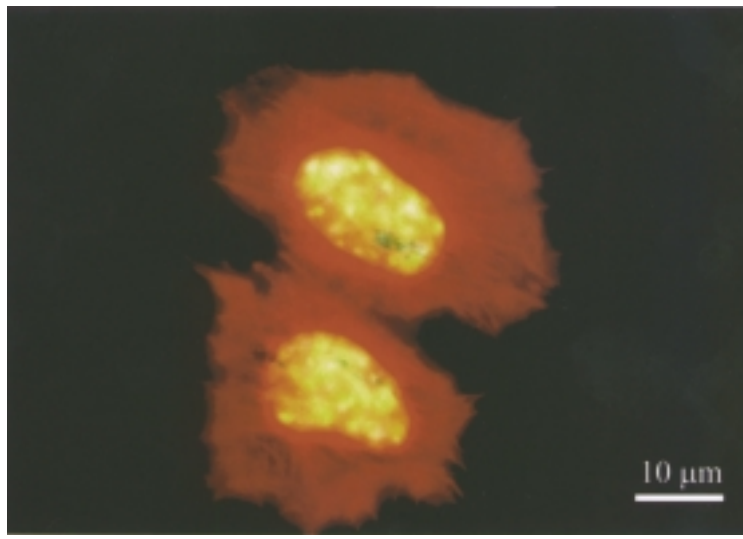

Fig.10
L929 cells immediately after irradiation:
double staining for actin (red) and DNA (blue)

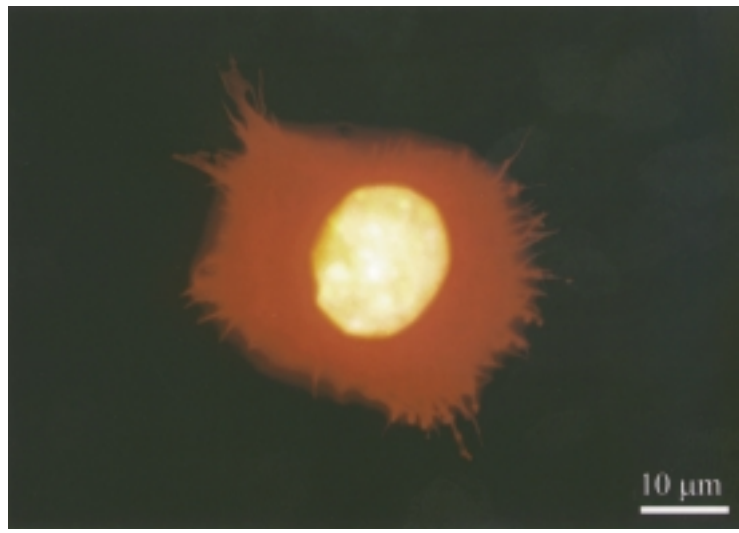

Fig.11
L929 cell at 30 min after irradiation:
double staining for actin (red) and DNA (blue)

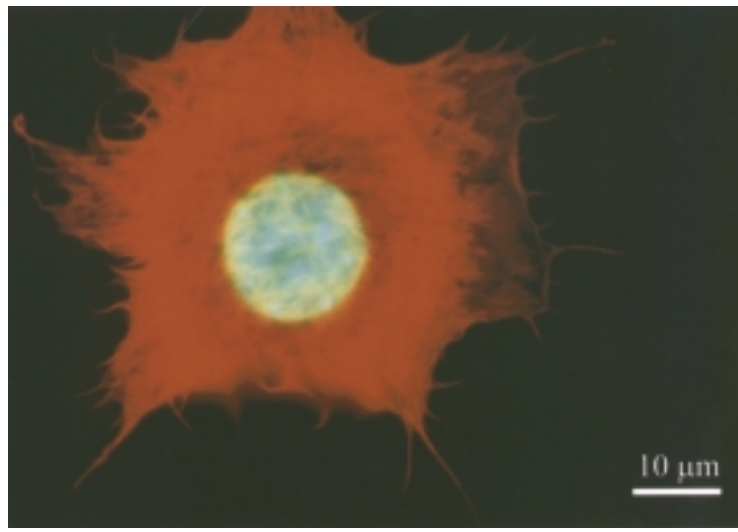

Fig.12
L929 cell at 3 h after irradiation:
double staining for actin (red) and DNA (blue)

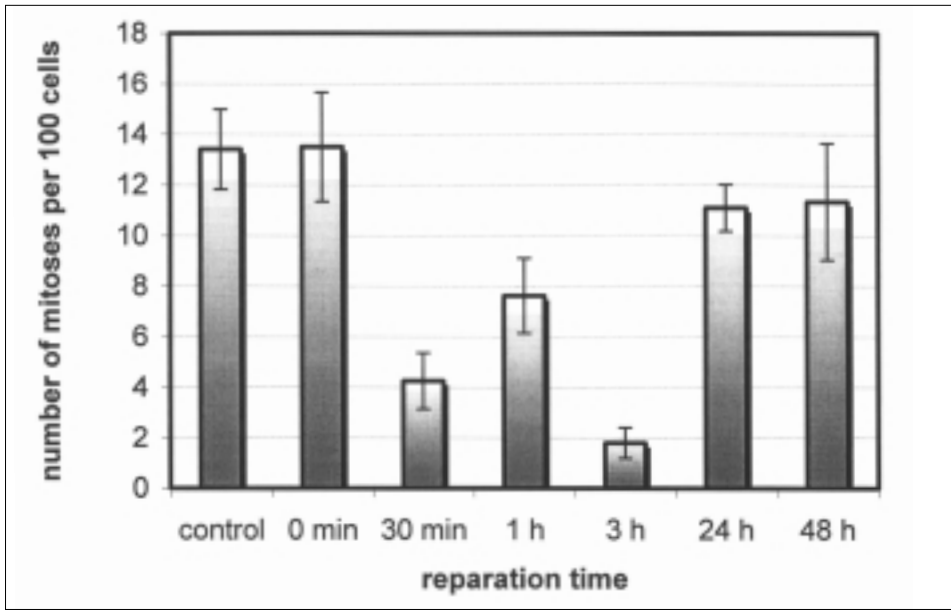

Fig.13
Mitotic index (means ± SEM)

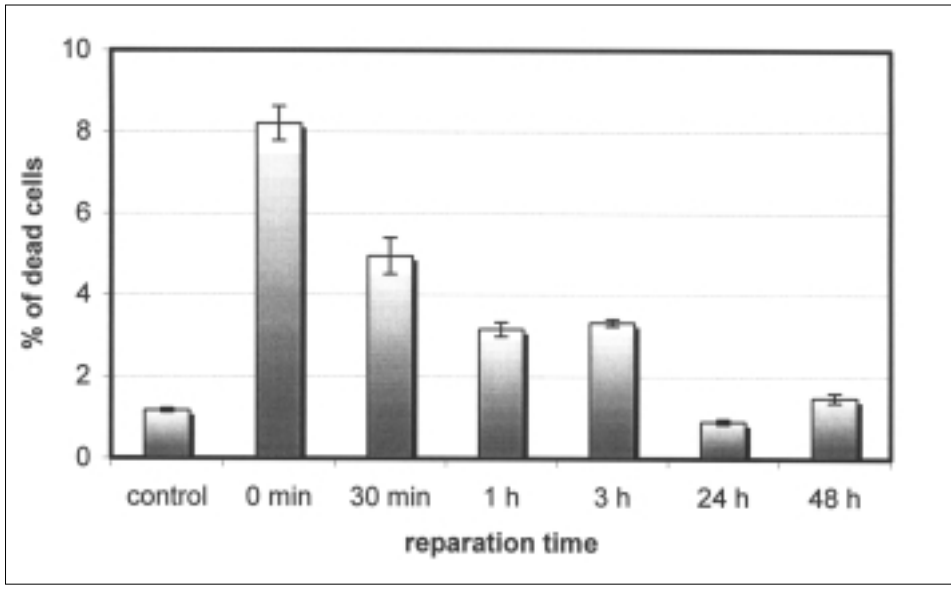

Fig.14
Cell viability (means ± SEM)

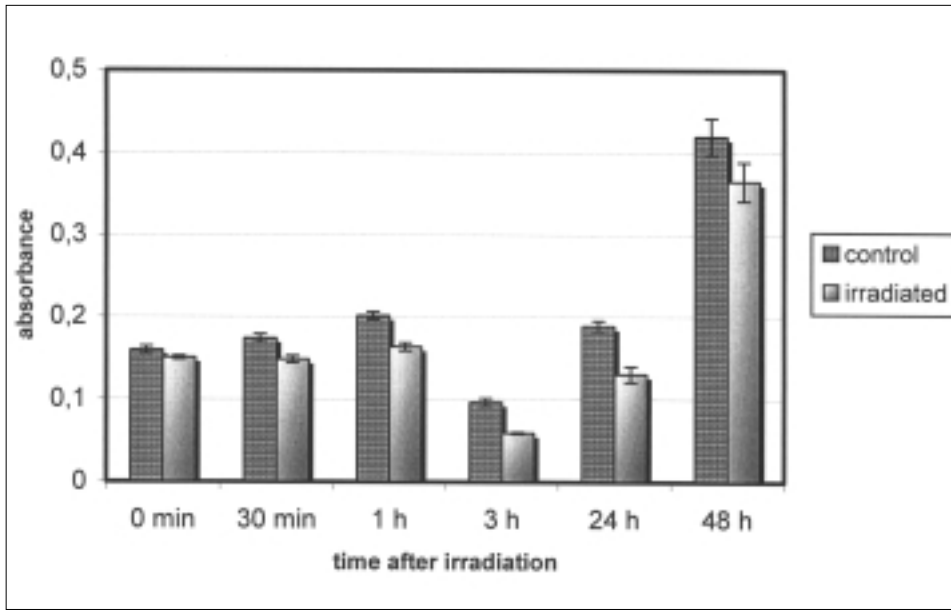

Fig.15
Proliferating activity (means ± SEM)

Proliferating activity

The diagram showing proliferating activities of cell populations is presented in Fig. 15. On the assumption that the starting cell concentrations were the same, there was a great difference in metabolic activity between the irradiated and the control cell populations immediately after irradiation. As time allowed for reparation process increased, the difference between the number of metabolically active cells in the irradiated population and that in the control population also increased. No lethal effect of UVR on the L929 cells was found during the periods investigated; UVR only caused a decrease in the total number of metabolically active cells together with keeping dynamics of cell population growth.

DISCUSSION

Our experiments showed that the type of UVA radiation at a wavelength of 356 nm distinctly decreased both viability and proliferating activity of the mouse

fibroblast L929 cell line. The irradiation led to disturbance of cell adherence, as well as to morphological changes and apoptosis induction. However, a part of the cell population survived and retained their reparation capacity. Although various changes in cell morphology were detected during the reparation process, 48 h after irradiation, the cell population had regained normal parameters.

This research was focused on cytoskeletal structures and their response to UVR exposure. Both microtubules and microfilaments showed different reactions to irradiation; microtubules were more sensitive to the type of UVR used than microfilaments. The integrity of microtubules was distinctly impaired due to irradiation. Individual microtubules were fragmented and the "thinning" of the microtubular network was evident, i.e., the total number of microtubules in the cell was reduced. The fragmentation of microtubules, noticeable during the short reparation periods, gradually disappeared and the integrity of microtubules was restored. Similar changes in the microtubular cytoskeleton have been described, for example, in a model of human epidermal cells, in which accumulation of microtubules in the perinuclear region was detected after UVB exposure (*5*). Analogous condensation of keratin intermediate filaments was observed in NHEK cells; in addition, tubulin rings were sporadically present (*11*).

In our experiments, microfilaments showed no marked damage to their integrity; only some structural abnormalities were detected during the process of reparation. This is in contrast with the data published on various kinds of human cells *in vitro* after exposure of the cells to both UVA and UVB radiation. Generally, either UVA or UVB irradiation have produced marked changes in the actin cytoskeleton, i.e., the loss of stress fibres and aggregation of microfilaments in the peripheral regions of the cells were detected in human epidermoid cells as well as in NHEK cells (*4*); the formation of actin rings and the presence of short, atypical microfilaments perpendicular to the rings were described in NHEK cells (*11*). Similar alterations in microfilament structure and defects in actin subunit polymerisation have also been reported in human fibroblasts exposed to UVA irradiation (*1*).

To summarize, the results obtained show that UVA radiation at the wavelength used in our experiments has a negative effect on both the individual cell morphology and the growth parameters of the whole cell population. The UVA radiation applied also induced distinct changes in the cytoskeletal structures studied, i.e., microtubules and microfilaments, with the microtubules being more sensitive than the microfilaments. In comparison with the findings reported on microtubules in other types of cells, especially human cell lines, the microtubules of L929 cells seemed to be more sensitive; however, the changes in the morphology of microtubules were similar to those in the other cell lines.

ZMĚNY CYTOSKELETU A ŽIVOTASCHOPNOSTI MYŠÍCH FIBROBLASTŮ LINIE L929 V DŮSLEDKU UV OZÁŘENÍ

S o u h r n

Předkládaná práce se zabývá vlivem dlouhovlnného ultrafialového záření na přežívání a integritu dvou hlavních cytoskeletálních komponent – mikrotubulů a mikrofilament – v myších fibroblastech linie L929 *in vitro* v závislosti na době reparace. K ozařování byl použit zdroj UV oblasti A o vlnové délce 356 nm; buňky byly ozářeny jednotnou dávkou UV-A, následně znovu kultivovány a postupně v určitých časových intervalech odebírány k dalšímu zpracování. Hodnocení morfologie cytoskeletu, stanovení mitotického indexu i testy životaschopnosti buněk byly prováděny pomocí fluorescenční mikroskopie. Proliferační aktivita buněk po ozáření byla měřena spektrofotometricky s využitím NRU-assay. Bezprostředně po ukončení ozařování byly v buňkách zaznamenány výraznější poruchy integrity mikrotubulů, ovšem bez vlivu na celkovou morfologii buněk. Po delších reparačních intervalech je pozorován výraznější nárůst buněk s příznaky, typickými pro indukovanou apoptózu (blebbing plazmatické membrány, fragmentace jaderné DNA). I u přežívajících buněk jsou zaznamenány charakteristické morfologické změny (zakulacení buněk, tvar tzv. „ozubených kol“ v případě buněk vícejaderných). S postupující dobou reparace se zvyšuje procento přežívajících buněk a klesá podíl buněk apoptotických (proliferace životaschopných buněk, odloučení mrtvých buněk do média). V reparujících se buňkách jsou patrné určité anomálie cytoskeletálních komponent, projevující se zejména atypickým průběhem jednotlivých vláken a jejich asymetrickým rozložením vzhledem k jádru. Po 48 h reparace již buněčná populace vykazovala zcela normální parametry životaschopnosti i morfologie jednotlivých cytoskeletálních komponent.

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