THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

The effects of the ultraviolet B radiation and other genotoxic agents on the chromatin structure of mammalian cells

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INTRODUCTION

The major part of DNA in the eukaryotic nucleus is packaged in chromatin with a compact conformation. For genes to be expressed the opposite process, decondensation of chromatin has to take place to accommodate transcription factors and RNA polymerase. For the study of gene expression the human erythroid leukemia K562 cell line was chosen as an adequate model in a transcriptionally active system. Understanding of the higher order folding and chromosome condensation in mammalian cells did not change significantly since it had been established in the 1970s. The existing models of chromosome condensation are contradictory and hypothetical because there are no methods, which would permit manipulations with the extremely polymeric, dense and sticky chromatin material. Due to the same technological limitations, methods to follow large scale chromatin changes do not exist.

To resolve this problem, we have introduced a new technique that is based on reversible permeabilization to maintain cell viability and replicative DNA synthesis. Replication has been studied in permeable cells as well as chromatin condensation in the interphase nucleus after reversal of permeabilization. Reversal of permeabilization was exploited to observe the nucleus in interphase in the absence of nucleotide incorporation and to visualize chromatin folding intermediates in synchronized cells, providing insight into the successive steps of the condensation process. While maintaining the viability of cells, this method allowed us to open the nucleus at any time during cell cycle. Using this approach, our studies in a variety of cell types with different chromosome numbers (Chinese hamster ovary (CHO), Indian muntjac, murine pre-B, and human erythroleukemia K562 cells) showed that chromosome condensation follows a previously unknown common pathway that includes several intermediate chromosome structures. Besides revealing intermediates of the normal supranucleosomal organization of DNA we have also shown structural aberrations in chromatin organization upon genotoxic treatments. Abnormal chromosomal forms were found after cadmium treatment and gamma irradiation leading to apoptosis. Cadmium treatment is known to cause disruptions and large holes on the nuclear membrane. Experiments with gamma irradiated cells revealed that ionization generates apoptotic bodies the size of which depends on the cell cycle, with many small apoptotic bodies in early S phase. Larger but less numerous chromatin bodies were seen at the end of S phase, correlating with the observation that in the most dense chromatin structure, the metaphase chromosomes, ionization leading to DNA breaks did not cause significant visible changes.

Ultraviolet B radiation is known to be a potent agent for the induction of programmed cell death. Yet, the mechanistic aspects of UV-induced apoptosis remain ill-defined. Human leukemia HL-60, myelomonocytic U937, T-lymphoblastoid Molt-4 and Molt-3 cells were found to rapidly undergo apoptosis after short periods of UV-irradiation, whereas prolonged exposure to UV radiation induced a more rapid form of cell death which was suggestive of necrosis. Pre-erythroid K562 and B-lymphoblastoid Daudi cell lines proved to be more resistant to the death-inducing properties of UV-irradiation by comparison. DNA fragmentation pattern indicative of endogenous endonuclease activation was not detected immediately after UV-irradiation. Lymphoid cells showed moderate damage after UV-irradiation, as assessed by DNA double-stranded breaks, while normal granulocytes and myeloid leukemia blasts expressed a sharp increase in double-stranded breaks. Ultraviolet rays induced chromosomal giant DNA fragmentation, which was followed by internucleosomal DNA fragmentation associated with apoptosis in rat glioma cells. Degradation into an oligonucleotide ladder appeared as early as 2 h after UV irradiation in hybridoma cells.

These results, especially the observation that lethal doses of UV irradiation generate high molecular weight (100–800 kbp) DNA fragments prior to ladder-formation of internucleosomal DNA fragmentation associated with apoptosis through caspase activation indicate the involvement of large scale chromatin changes in DNA structure induced by UV irradiation.

In this thesis we describe biochemical and morphological changes taking place in regenerating non-irradiated K562 cells and in cells exposed to low (2 and 5 J/m^2), moderate (15 J/m^2) and high (25 J/m^2) doses of ultraviolet B (UVB) irradiation after reversal of permeabilization. Biochemical measurements showed partial recovery of replicative DNA synthesis at lower and moderate UVB doses and stagnant suppression of DNA replication at high UVB dose. Large-scale chromatin changes generated by ultraviolet (UV) B irradiation were visualized throughout the cell cycle. These preapoptotic chromatin changes preceded the apoptotic disintegration of the nuclear material.

AIMS

Our aims were to follow biochemical and morfological changes in response to UVB irradiation using the human erithroleukemia K562 cell line, as well as to systematize and compare the different structures that developed upon genotoxic agens.

1. We examined biochemical changes after UVB irradiation (at the level of DNA synthesis)

2. Our 2nd major aim was to characterize identifiable and the specific chromatin forms-typical to UVB irradiation.

3. We have observed the temporal appearance of the apoptotic changes in a cell cycle-dependent manner.

4. The deleterious effect of the UVB and UVC radiation have been compared.

5. The differences in chromatin srtuctures and chromatin condensation pattern after gamma-irradiation, cadmium treatment and that of UVB irradiation have been described.

6. Genotoxic „fingerprint" was done on the basis of the characterization and comparison of chromatin structures, isolated from human erithroleukemia K562 cells after gamma, UVA, UVB, cadmium, mercury, nickel, lead threatment.

MATERIALS AND METHODS

The isotopes [^{3}H]-thymidine 5'-triphosphate (3.03 TBq mmol^{-1}) and [^{3}H]-thymidine (2.86 TBq mmol^{-1}) were purchased from ICN Isotope and Nuclear Division (Irvine, CA, USA). 2,6-diamino-2-phenylindole (DAPI) was the product of Braunschweig Chemie (Braunschweig, Germany). Dextran T-150 was purchased from Pharmacia- Biochemicals (Gaitersburg, MD, USA). Colcemid (N-methyl-N-deacetyl-colchicine) was supplied by Boehringer (Mannheim, Germany). Growth media and sera were obtained from Invitrogene (Carlsbad, CA, USA). Nucleotides, 1,4-diazobicyclo-(2,2,2)-octane and other reagents were from Sigma-Aldrich Chemie GmbH (Munich, Germany).

Antifade medium consisted of 90% glycerol, 2% (w/w) 1,4-diazobicyclo-(2,2,2)-octane, 20 mM Tris–Cl, pH 8.0, 0.02% sodium azide and 25 ng/ml DAPI for blue fluorescent total staining of DNA or of 0.2 µg/ml propidium iodide for red DNA staining. Isotonic buffer contained 140 mM sucrose, 60 mM KCl, 10 mM Hepes, pH 7.4, 5 mM KPO_4, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$. Hypotonic buffer for reversible permeabilization contained 9 mM HEPES, pH 7.8, 5.8 mM dithiothreitol, 4.5% dextran T-150, 1 mM EGTA and 4.5 mM $MgCl_2$. Swelling buffer consisted of 50 mM KCl, 10 mM $MgSO_4$, 3 mM dithiothreitol and 5 mM $NaPO_4$, pH 8.0.

Cell growth and ultraviolet irradiation

Human erythroleukemia K562 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). The continuous cell line K-562 was established by Lozzio and Lozzio from the pleural effusion of an 53-year-old female with chronic myelogenous leukemia in terminal blast crises. We have used UV-B (280 nm) for irradiating cells, which is part of the direct solar UV-B (280–320 nm) radiation and is strongly absorbed in the stratosphere by ozone, but increased through the ozone holes. For UV irradiation cells were grown for 15 h, rinsed with PBS and exposed to 2–25 J/m^2 ultraviolet B (UVB) (280 nm) irradiation at cell density of 10^7 cells/ml in PBS. Cells were grown in RPMI-1640 medium supplemented with 10% FBS for an additional time as indicated in the experiments before being harvested.

Elutriation of cellular fractions

Based on the relationship between cell size and DNA content cells were separated by centrifugal elutriation and several fractions were collected. The cells within each fraction belonged to the same cell cycle phase, i.e. they were synchronous in each elutriation fraction. Fractionation was performed by counterflow centrifugal elutriation. Cells were grown for 15 h to a final concentration of 2.4 x 10^5 cells/ml, harvested by centrifugation at 600g for 5 min at 5°C, collected in 20 fractions and resuspended in RPMI 1640 medium containing 1% FBS at 10^7 cells/ml. Cells (1.8 x 10^8) were introduced into the elutriator rotor (Beckman J-6 MI) equipped with a JE-5.0 elutriation system including a Sanderson chamber (Beckman Instruments, Inc., Palo Alto, CA) and a MasterFlex peristaltic pump (Cole-Parmer Instruments). Elutriation was performed at 20°C and 20 cell fractions (100 ml each) were collected by elution in RPMI medium containing 1% fetal bovine serum at increasing flow rates. The fraction collected during loading, which contained dead cells, was discarded. The last fractions (fractions 19–20) could not be used either, due to their heterogeneity and low cell number. Each fraction was routinely monitored by light microscopy and even fractions were analyzed by fluorescence activated cell sorting (FACS). The fractions contained unaggregated cells, which increased in size with each fraction elutriated. Cell number, size and volume were determined. Cell number was also assessed in a Burker chamber and viability (>98%) was determined by trypan blue dye exclusion in each fraction. All experiments were repeated three times with similar results.

FACS analysis of cell cycle

Cells were fixed with 70% methanol at room temperature and stained with 50 µg/ml propidium iodide (Sigma). The cells were analyzed by a FACS can flow cytometer (Becton Dickinson) using the CellQest (Becton Dickinson) software as described. The nuclear DNA content, expressed in C values (1 C value corresponds to a haploid DNA content per cell) increased from 2 C to 4 C and provided a measure of progression through the S phase. Flow cytometric profiles giving the distribution of DNA content were used to calculate the average C-value for each elutriated fraction. The cells in each fraction were divided into 17 subfractions to cover the total area of the cytometric profile. C-values were calculated from

the appropriate area under the flow cytometric profile and were averaged to yield the DNA content for each fraction as published previously.

Reversible permeabilization

This method, originally developed for the reversible permeabilization of murine lymphocytes was adapted to human K562 cells. Briefly, 1 ml of hypotonic buffer was added to 10^6 cells in the presence of Dextran T-150 as a molecular coat to prevent cells from disruption. Permeabilization was carried out for 2 min at 0°C. For the reversal of permeabilization, the hypotonic solution was replaced by RPMI 1640 medium containing 10% fetal bovine serum, and the cells were incubated in a CO_2 incubator at 37°C and 5% CO_2 for 3 h.

ATP-dependent replicative DNA synthesis in permeable cells

Cells (10^6) were permeabilized and replicative DNA synthesis was assayed as described. The mixture (100 µl) contained 100 mM HEPES pH 7.8, 210 mM NaCl, 15 mM ATP, 0.3 mM dATP, dGTP, and dCTP, 0.01 mM dTTP, 37 kBq [^{3}H]dTTP (3.03 TBq mmol$^-$), 5.8 mM dithiothreitol (DTT), 2 mM $MgCl_2$, and 0.2 mM $CaCl_2$. After 10 min of pulse label at 37°C, the reactions for DNA synthesis were stopped by the addition of 1 ml 5% TCA. After standing in ice for 1 h, the precipitates were washed on glass fiber filters and counted for TCA insoluble radioactivity.

[^{3}H]thymidine incorporation in intact and permeable cells

Intact or permeable K562 cells (10^6) were suspended in 0.5 ml RPMI medium and incubated in the presence of 37 kBq [^{3}H]thymidine (2.86 TBq mmol^{-1}) at 37°C for 10 min. The reaction was stopped by the addition of an equal volume of 5% TCA. After standing in ice for 1 h, the precipitates were washed on glass fiber filters and counted for TCA insoluble radioactivity.

Isolation of nuclei

On account of the cyclic character of chromatin unfolding and chromosome condensation, which is limited to a relatively short time, synchronized cell populations were treated with colcemid to arrest the cycle in metaphase. Cells (10^6) were resuspended in growth medium after reversal of permeabilization and treated with 0.1 µg/ml colcemid for 2 h at 37°C under 5% CO_2. Cells were washed with PBS and incubated at 37°C for 10 min in swelling buffer, followed by centrifugation at 500g for 5 min. Nuclei were isolated by the slow addition of 20 volumes of fixative (methanol:glacial acetic acid, 3:1) and were then centrifuged at 500g for 5 min, washed twice in fixative and resuspended in 1 ml of fixative. Cellular and nuclear volumes and nuclear diameter were determined with the Coulter Multisizer.

Spreads of nuclear structures

We used a method developed for metaphase chromosomes to prepare nuclear spreads. Nuclei were centrifuged at 500g for 5 min, washed twice in fixative and resuspended in 1 ml fixative. Nuclei were spread over glass slides dropwise from a height of approximately 30 cm. Slides were air dried, stored at room temperature overnight, rinsed with PBS and dehydrated using increasing concentrations of ethanol (70, 90, 95 and 100%).

Visualization of chromatin structures

Dehydrated slides containing isolated chromatin structures were mounted in 35 µl antifade medium under 24 x 50 mm coverslips. Blue fluorescence of DAPI was monitored with Olympus AX70 fluorescence microscope.

Time-lapse photography

Two inverse microscopes sitting in CO2 incubator were equipped with high sensitivity video cameras, connected to a custom-built dual image acquisition computer system. Custom-built illumination was developed to minimize heat- and photo-toxicity. Operation of the spectrally cold-white light emitting diodes were synchronized with image acquisition periods.

Cell cultures in T-flasks were placed on inverse microscopes. The screen of the computer was divided in two portions showing side-by-side the morphological changes of the control and the treated cells. Photographs of K562 cells were taken every minute. The time of exposure was indicated in each frame. Exposures were converted to videofilms by speeding up the projection to 30 exposures/s. Individual K562 cells of suspension cultures were selected for further analysis. Individual photographs were chosen as panels shown in the figures. Time-lapse photography of individual cells allowed us to determine the growth profile of individual cells grown both in monolayer and in suspension cultures. Genotoxic specific growth profiles of cell death could be distinguished.

RESULTS

A major obstacle in the attempt to follow DNA synthesis was the impermeability of the cell membrane to large molecules such as nucleotides, cyclic nucleotides and proteins. Thus many studies with intact cells used intermedier metabolites (e.g. thymidine, uridine) which undergo active transport and several steps before being incorporated into macromolecules. It was originally described in bacteria that DNA synthesis in permeable cells depends on the presence of ATP, characteristic of replicative DNA synthesis. The ATP dependent incorporation of the four deoxyribonucleoside triphosphates (dNTPs) in permeable thymocytes reflected DNA replication and was hampered by inhibitors of DNA replication, while dNTP incorporation in the absence of ATP could be prevented by inhibitors of repair synthesis. These experiments served as a basis to follow the two types of DNA synthesis and chromatin condensation in reversibly permeabilized K562 cells. Before going into experimental details a scheme is given below.

We have used non-irradiated and UV-irradiated cells to test:

(A) Biochemical changes including:

– viability of intact, non-irradiated cells

– regeneration of non-irradiated cells after permeabilization

  – recovery of [^{3}H]thymidine incorporation

  – decreasing incorporation of [^{3}H]TTP

– the absence of apoptotic morphology at cellular level in irradiated cells after reversal of permeabilization

– regeneration of UV irradiated cells after reversal of permeabilization

  – dose dependent recovery of [^{3}H]thymidine incorporation

– DNA content during the cell cycle of non-irradiated and irradiated populations of synchronized cells

– ATP-dependent replicative and ATP-independent repair DNA synthesis

  – replicative synthesis in non-irradiated cells

  – repair synthesis in non-irradiated cells

  – replicative DNA synthesis in irradiated cells

  – repair synthesis in irradiated cells

(B) Morphological changes:
– interphase chromatin structures in non-irradiated cells
– interphase chromatin structures in irradiated cells
– final stage of chromosome condensation in non-irradiated and irradiated cells

Cell viability and DNA synthesis in permeable cells

The viability of intact cells was measured in the the trypan blue exclusion test and was expressed as percentages of the total cells. At the beginning of the experiment less than 2% of intact K562 cells were able to take up trypan blue dye molecules. After 10 h of growth we counted approximately 5% less viable cells in the same control population. Human erythroleukemia K562 cells were made permeable with hypotonic buffer in the presence of 4.5% dextran T-150. K562 cells became permeable rapidly, more than 95% of the cells took up trypan blue within 5 min. Simultaneously, [^{3}H]thymidine incorporation was measured in permeable cells. We followed the kinetics of cell regeneration and found that reversal of permeabilization was fast at the beginning and slowed down after 4 h. Permeable cells which were originally unable to incorporate [^{3}H]thymidine, gradually regained their capacity to incorporate the radioactive nucleoside. After 10 h approximately 80% of the cells regained their membrane integrity. The opposite tendency was observed when [^{3}H]dTTP incorporation was measured. Permeable cells gradually lost their ability to incorporate the four deoxyribonucleotides (dNTPs) including [^{3}H]dTTP.

Effect of ultraviolet irradiation on replicative and repair DNA synthesis in permeable cells

The rate of DNA synthesis of fibroblasts was inhibited by approximately 60% after irradiation with 5.2 J/m^2 of 254 nm UV light. This observation served as an orientation point to determine the radiosensitivity of K562 cells. Since K562 cells have lower radiation sensitivity, higher UV doses have been applied. Exponentially growing human K562 cells treated with 15 J/m^2 280 nm ultraviolet light at 10^5 cells/ml cell culture density were grown further for 3 h, then permeabilized. After these treatments 65% inhibition of ATP-dependent replicative DNA synthesis was measured. Repair synthesis was measured in the absence of ATP. The ATP independent repair synthesis after UV irradiation was almost as high as the

ATP-dependent replicative synthesis in non-irradiated cells indicating that UV treatment induced DNA repair. The difference between the ATP-dependent replicative synthesis and ATP independent repair synthesis after UV irradiation clearly indicates, that replication is almost completely blocked after irradiation and the measured rate of residual DNA synthesis can be attributed to DNA repair.

Flow cytometric analysis after UV irradiation

Exponentially growing K562 cells were subjected to increasing fluencies of UV irradiation (6, 12 and 24 J/m^2). Irradiation of cells was followed by incubation in growth medium for further 3 h. The lack of small apoptotic cells showed that apoptosis did not develop in such a short period of time after irradiation.

Restoration of nucleoside incorporation after irradiation in intact cells

K562 cells (10^6) were irradiated with increasing doses of UV light from 2 J/m^2 to 25 J/m^2. [^{3}H]Thymidine ([^{3}H]dThd) incorporation was measured in intact non-irradiated cells and after irradiation. The UV dose dependent decrease of DNA synthesis was measured. The rate of DNA synthesis of cells irradiated with lower (2 and 5 J/m^2) doses of UV light showed a gradual recovery after 3 h. Higher dose of UV irradiation (15 J/m^2) could be partially overcome (80%) by regenerating cells, while cells exposed to high dose of UV light (25 J/m^2) were unable to regenerate their DNA synthesizing capacity.

Cell cycle analysis in intact and UV irradiated cells

The flow cytometric profiles of even fractions of elutriated non-irradiated cells were averaged to assay the DNA content. The DNA content of exponentially growing non-irradiated cells increased from 2.07 to 3.88 C-values. Typical chromatin structures belonging to fractions isolated from non-irradiated cells will be described in the next paragraph. The separation of irradiated cells (24 J/ m^2) was carried out in the same way, but their C-values could not be determined due to their variation. The estimated C-values of fractions of irradiated cells ranged between 2.2 and 2.4 C-values, corresponding to fibrillary chromatin structures seen in non-irradiated cells.

Interphase chromatin structures in non-irradiated cells

Reversible permeabilization was used to restore cellular membranes which allowed to open the nucleus throughout the cell cycle. Control experiments included cells: (a) without permeabilization, (b) after permeabilization without reversal of permeabilization as described earlier. The gradual change in shape of chromatin structures from 2.0 to 3.7 C-values is summarized (Elutriation fractions 1–16). Decondensed chromatin appears as a veil-like fibrillary structure (fraction 1–2), which becomes polarized with oval or elongated shapes (fraction 3–4). Elongated chromatin gradually turns to supercoils (fractions 5–6). The fibrillary structure changes to fibrous forms (fractions 7–8) showing the linear connection between early decondensed chromosomes (fractions 9–10). Supercoiled loops form chromatin bodies, which are regarded as the earliest visible forms of interphase chromosomes (fraction 11–12). The continuity is maintained in precondensed bent chromosomal forms (fraction 13–14). Bent forms open up to linear structures, which did not reach the compactness of metaphase chromosomes (fraction 15–16).

Changes in chromatin structure after UV irradiation

Chromatin changes occurred only occasionally at lower doses of UV light (6, 12 J/m^2), and due to the infrequency we did not visualize them. High UV light dose (24 J/m^2) manifested mainly as an increased fibrillary cloud covering the condensing chromatin structure. As a result all chromatin structures became blurred. At the early unset of S-phase fibrillary structures are more decondensed than in the same fraction of non-irradiated cells and polarization of chromatin starts earlier (fraction 1–2). Elongated chromatin forms appear earlier (fractions 3–4), but the elongation process seems to be arrested (fraction 5–6). Fibrous structures covered with a fine fibrillary network dominate the pictures from fraction 5 without significant progression in supercoiling which would lead to more compact structures. Although, small apoptotic bodies were observed occasionally (fraction 9 and 10), but contrary to gamma irradiation they are not typical structures of UV irradiation. Neither chromatin bodies, nor other typical chromosomal forms could be observed after UV irradiation. Primitive, early forms of chromatin condensation were covered with faint fibrillary chromatin. Chromatin condensation did not reach the stage of visible chromosomes (fractions 11–16).

Final stage of chromatin condensation

Fluorescent images of chromatin structures of the last elutriation fractions (fraction 18) were compared in non-irradiated and irradiated cells. In fraction 18 of non-irradiated (normal) cells chromatin condensation reached its final stage, occasionally metaphase. After UV irradiation (24 J/m^2) only partial segregation of chromosome domains could be seen, fully condensed chromosomes were not visible. The stickiness of the fine fibrillary network covering the incompleteley folded primitive chromosomes is assumed to prevent the opening of the nucleus.

Comparing the effects of UVA and UVB irradiation

The consequences of different doses of UVB are similar to the UVA treatment, where the obstructive effect of the irradiation is dependent to the duration of the treatment. Lower than 25 J/m^2 doses (3, 6, 12,5 J/m^2) have inhibitory effect, from which most of the cells were able to revover. To the contrary after 25 J/m^2 irradiation the cells were not able to regenerate and obviously underwent apoptosis. This led us to the conclusion that the short time of exposure either suspended the progression of the cell cycle or retarded apoptotic death. In accordance with this hypothesis the injury of the genotoxicity may extend the time of apoptosis. It is remarkable that the UVA which has higher wave-length intervallum but lower power of irradiation, nevertheless can generate morfological changes at lower dosage contrary to UVB radiation. To explain this phenomenon the repair system may have been evolved primarily to the repair damages caused by UVB. A further aim of the research group will be to explain the synergistic effects of UVA and UVB during the development of epithelioma and to characterize those factors that play central role in cromosome condesation.

Comparison of effects of UVB and gamma irradiation

Preapoptotic changes upon γ-irradiation in CHO cells and during UVB in K562 cells were seen as: a) increase in cellular and nuclear size, b) the DNA content was lower in each elutriated subpopulation of cells, c) the progression of the cell cycle was arrested in the early S phase at 2.4 C value, and between 2.2-2.4 C value upon UVB irradiation, d) the chromatin

condensation was blocked between the fibrillar chromatin and precondensed elongated chromosomal forms.

The main difference between the UVB and gamma irradiation was the emergence of the apoptotic bodies after gamma irradiation. The number and size of apoptotic bodies were inversely correlated with the progression of the cell cycle. In case of the gamma irradiation many small apoptotic bodies were observed in early S phase and less but larger apoptotic bodies in late S phase, whereas after UVB treatment insignificant amount of apoptotic bodies became visible. Similar observations were made in K562 cells after gamma irradiation.

Chromatotoxic fingerprinting

Since the potential to distinguish among different chromatotoxic effects is of diagnostic significance, we have started to determine and systematize the effects of cadmium treatment and gamma irradiation. After these results we have carried out further experiments with other heavy metals (Hg, Pb, Ni). Identical cell density ($1x10^6$/ml) in logarithmic phase, and the same conditions were used.

Cadmium chloride

Genotoxic treatments may have multiple effects on different cell lines, therefore we have used two cell lines (Chinese hamster ovary cells and murine preB cells) for cadmium treatment and have seen the same large extensive disruptions and holes in the nuclear membrane and sticky incompletely folded chromosomes typical for cadmium treatment. We have noticed, that the apoptotic bodies witch are similar to precondesed chromatins, already appear in the early S phase. The polarised nuclear material is distinctly visible contrary to control cells, and this process leads to the development of chromatin bodies in this stage of the cell cycle. In the middle of the S phase the formation of chormatin fibers and bodies appear wich are in contrast to the normal intermediers. In the late S phase we have seen large extensive disruptions and holes in the nuclear membrane, besides the sticky incompletely folded chromosomes, that are typical for cadmium treatment. We have extended the morfological experiments tó other heavy metal-treatment.

Mercury acetate

With the aid of our custom built Long Term Scanning system we have folowed the fate of individual cells after treatment with 10, 20, 100, 150, 200, 1000 μM conentrations of Hg(II) acetate. We have isolated chromatin structures from reversibly permeabilized cells

pretreated with subtoxic (1 μM) and toxic concentration of mercury. Upon subtoxic dose the uneven condensation of chromatin was indicated by the polarization of dense fluorescent chromatin patches, which tended to reject the less dense fibrillar chromatin or apoptotic chromatin bodies from the nuclear material. Upon treatment of K562 cells with low (10-50 μM) mercury acetate the gradual aggravation of apoptosis could be visualized. In the presence of 10 μM mercury acetate the polarization of chromatin, the appearence of holes in the nucleus, as well as the disintegration of the nuclear material and apoptotic body formation are dominated in the pictures. The different size of apoptotic bodies seems to be related to the cell cycle status with many small decondesed apoptotic bodies in early S phase and less but more condensed bodies in late S phase. After treatment with somewhat higher Hg(II) acetate concentration (15 μM) not only tha cell size but also the nuclear size decreased during these apoptotic changes. At higher mercuric concentration (100 μM) necrotic chromatin changes have been observed with enlarged nucleus and changes in the isolated nuclear material. The generation of large holes were noticeable inside the nucleus, however the nucleus did not fall apart and its ring structure was maintained. The opening of the nucleus revealed that the nuclear material has two ends and the chromatin is a continuous structure. Occasionally the chromatin was broken down to many small particles, with the round shape of the nucleus remaining visible.

Lead nitrate

After 2-5 μM concentration of lead nitrate treatment rounded ribbond chromatin forms were visible in the early S phase. In the middle of the S phase fibrillar chromatin, thin and thick chromatin fibers could be observed. In the late S and M phases the aggregation of the nucleus is typical, wich leads to abnormal cytokinezis. Moreover, the disintegrated nuclear membrane, released nuclear material, with enormous extracellular pericromatic granules, and the presence of elongated prechromosomes became visible. After 10, 20, 40 μM concentration of $Pb(NO_3)_2$ treatment in the early S phase apoptotic bodies, in the middle of the S phase chromatin ribbons, in late S phase precondesed chromosomes dominated the picture. Sticky nuclei tended to adher to each other. The rounded, polarised, dense, strongly fluorescenced marginal patches of the chromatin were distinctive features of the changes caused by lead nitrate.

Nickel chloride

The treatment with as small as 1 μM concentration of nickel chloride already caused disorders in supercoiling, deformation in the ribboned chromatin and induced apoptosis. At 5

μM $NiCl_2$ the presence of early veiled structures could be explained by the interruption of the fibrillary chromatin. At 10 μM concentration apoptotic bodies appeared and the release of the faint nuclear material dominated. Strongly polarised dense substance and veiled, decondensed forms appeared simultaneously. At 50 μM the necrotic enlarged nucleus was the typical feature.

Cellular etology and cell viability tested with the LTS system

We have performed heavy metal treatment in K562 cells in logarithmic phase at 10^6/ml cell density, simultaneously a control culture was grown. During the 24 h observation photographs of K562 cells were taken every minute. Exposures were converted to videofilms by speeding up the projection to 30 exposures/s. Individual K562 cells of suspension cultures were selected for further analysis. Genotoxic specific growth profiles of cell death were expected to be distinguished. Generally features were the interruption of the cell growth, degeneration of the cell membrane and the appearance of different forms of the apoptosis in the cell cultures. After heavy metal treatment the following differences were observed:

Lead nitrate: The initial low concentration of lead nitrate induced transitional increase in cell growth, while at higher concentrations obstructive and letal effect were measurable.

Mercury acetate: In case of the nickel chloride and the lead nitrate treatment the products of disintegration and the broken fragments of the cells were eliminated and taken up by the neighboring cells. The damaged cells after mercury acetate treatment remained visible, what attributed to the efficient, paralysing, fixative effect of this heavy metal

Nickel chloride: We observed an increased activity in cellular movement, compared tó the movement of the other two (Hg, Pb) treatments and to the control culture.

In the viability analysis we have observed similarly different results. The completion of the control cell cycle and the doubling of cell number took about 24 h. At the lowest mercury acetate contrentation (1 μM) the rate of the living cells has been 75 %, and the degree of the destruction has been relatively high, 37 %. At the highest concentration the letality was 100 %. We have noticed the same tendency of the toxicity by nickel chloride at the lowest concentration (1 μM), where the level of the mortality was 27 % and the cell number of living cells was 75 %. In case of lead nitrate the treatment (1 μM) the number of cells relative tó the initial culture has been increased . The degree of letality (15%) was as much as in the control cells (13%), to the contrary the degree of cell death (41%) was higher at the measured highest concentration (50 μM).

DISCUSSION

We have studied biochemical and morphological changes after UV irradiation in K562 cells.

1. Biochemical experiments aimed to prove that DNA synthesis in permeable K562 cells is:

(a) **an ATP-dependent process** (b) **and reversible permeabilization maintains cell viability**. Human erythroleukemia K562 cells were made permeable with hypotonic buffer in the presence of 4.5% dextran T-150. K562 cells became permeable rapidly, more than 95% of the cells took up trypan blue within 5 min. Simultaneously, [^{3}H]thymidine incorporation was measured in permeable cells. We followed the kinetics of cell regeneration and found that reversal of permeabilization was fast at the beginning and slowed down after 4 h. Permeable cells which were originally unable to incorporate [^{3}H]thymidine, gradually regained their capacity to incorporate the radioactive nucleoside. The opposite tendency was that the permeable cells gradually lost their ability to incorporate the four deoxyribonucleotides (dNTPs) including [^{3}H]dTTP.

(c) **UV irradiation inhibits replicative DNA synthesis and favours repair DNA synthesis**. Exponentially growing human K562 cells treated with 15 J/m^2 280 nm ultraviolet light at 10^5 cells/ml cell culture density were grown further for 3 h, then permeabilized. After these treatments 65% inhibition of ATP-dependent replicative DNA synthesis was measured. Repair synthesis was measured in the absence of ATP. The ATP independent repair synthesis after UV irradiation was almost as high as the ATP-dependent replicative synthesis in non-irradiated cells indicating that UV treatment induced DNA repair.

(d) **Flow cytometric analysis after UV irradiation** showed the presence of fibrillary chromatin in each fraction which made it impossible to dustinguish among DNA profiles.

(e) **UV irradiation and reversal of permeabilization do not immediately cause apoptotic shrinkage of cells**. Earlier studies on DNA replication in ultraviolet-irradiated HeLa cells have shown that the extent of inhibition varied with time. At doses less than 10 J/m^2 the rate was initially depressed but later showed some recovery. At higher doses, a constant, low rate of DNA synthesis was seen for at least 6 h. We confirm these observations, and the higher UV resistance of K562 cells. The lower UV doses which allowed recovery turned out to be 2, 5 and 15 J/m^2 for K562 cells. After these treatments the repair mechanisms of K562 cells were able to remove at least partially DNA damages, while at high dose (25 J/m^2) K562 cells were

unable to recover and deemed to undergo apoptosis. This probably means that short exposures or low concentrations of genotoxic agents cause delayed apoptotic death.

1. Morphological experiments show the following results:

(a) **The estimated C-values of fractions of irradiated cells ranged between 2.2 and 2.4 C-values**, corresponding to fibrillary chromatin structures seen in non-irradiated cells as opposed to the 2.07 to 3.88 C-values of the control cells.

(b) **In chromatin structure after UV irradiation at the early unset of S-phase fibrillary structures are more decondensed** than in the same fraction of non-irradiated cells and polarization of chromatin starts earlier (fractions 1-2) and elongated chromatin forms appear earlier (fractions 3–4), but the elongation process seems to be arrested (fraction 5–6). Fibrous structures covered with a fine fibrillary network dominate without significant progression in supercoiling which would lead to more compact structures. Although, small apoptotic bodies were observed occasionally (fraction 9 and 10), but contrary to gamma irradiation they are not typical structures of UV irradiation. Primitive, early forms of chromatin condensation were covered with faint fibrillary chromatin. Chromatin condensation did not reach the stage of visible chromosomes (fractions 11–16).

(c) **The UVA which has higher wave-length intervallum but lower power of irradiation, can generate morfology changes in lower dosis opposite to UVB radiation.** The short time exposure to low concentration of genotoxic agents retarded apoptotic death. In accordance with our hypothesis the injury of the genotoxicity extends the time of apoptosis. It is remarkable that the UVA which has higher wave-length intervallum but lower power of irradiation, can generate morfology changes in lower dosis opposite to UVB radiation. At the bottom of this phenomenon may stands the repair system, which is evolved primarily to repair the damage of UVB.

(d) **The main difference between the UVB and gamma irradiation is the emergence of the apoptotic bodies.** After gamma irradiation the number and size of apoptotic bodies were inversely correlated with the progression of the cell cycle. In case of the gamma irradiation many small apoptotic bodies are observable in early S phase and less but larger apoptotic bodies in late S phase, whereas after UVB treatment just insignificant amount of apoptotic body are visible. Further preapoptotic changes upon γ-irradiation in CHO cells were seen as: (a) The cellular and nuclear sizes increased. (b) The DNA content was lower in each elutriated subpopulation of cells. (c) The progression of the cell cycle was arrested in the early S phase at 2.4 C value. (d) The chromatin condensation was blocked between the fibrillar

chromatin and precondensed elongated chromosomal forms. (e) The number and size of apoptotic bodies were inversely correlated with the progression of the cell cycle, with many small apoptotic bodies in early S phase and less but larger apoptotic bodies in late S phase.

(e) **Morphological studies after genotoxic treatments suggested that the consequences of various chromatin injuries can be categorized based on the assessment of injury-specific chromatin changes.** In a broader sense our aim was to characterize external apoptotic changes caused by genotoxic agents and classify them according to their structural chromatin changes. Since the potential to distinguish among different chromatotoxic effects is of diagnostic significance, we have started to determine and systematize the effects of cadmium treatment, gamma irradiation. We have carried out further experiments with other heavy metals (Hg, Pb, Ni). Other compounds (EtBr, actinomycine, coffein, nicotin) have also been teseted and their chromatin specific changes are under investigation. Genotoxic treatments may have multiple effects on different cell lines, therefore we have used two cell lines (Chinese hamster ovary cells and murine preB cells) for cadmium treatment and have seen the same large extensive disruptions and holes in the nuclear membrane and sticky incompletely folded chromosomes typical for cadmium treatment.

(f) **After the examination with LTS assay the general features of apoptosis are the interruption of the cell growth, degeneration of the cell membrane and the appearance of different cellular forms.** Differences of the results: Lead nitrate: The initial low concentration of lead nitrate induced transitional increase of cell growth, later the obstructive and letal effect are measurable.

Mercury acetate: While in case of the nickel chloride and the lead nitrate the products of disintegration and the broken fragments of the cells are eliminated and digested by the sourronding cells, the decomposed cells treated with mercury acetate remained visible, which is attributed to the paralysing, fixative effect of this agent. Nickel chloride: Upon the experiment we observed fairly active cellular movement, compared to the movement of cells treated with the other two heavy metals.

SUMMARY

The aim of our experiments was the visualization of chromatin changes taking place in human erythroleukemia cells during the interphase upon UVB irradiation and other genotoxic agents, to compare the different structures, apoptotic features and to categorize them.

Two different approaches were used to answer the questions, involving biochemical and morphological studies.

We have provided evidence that the reversible permeabilization that allowed the visualization of chromatin structures of interphase nuclei did not reduce significantly the viability of cells and that DNA synthesis taking place in permeable K562 cells is an ATP dependent process. UVB irradiation inhibited replicative DNA synthesis and activated repair synthesis. Our flow cytometry measurements proved that small apoptotic cells did not appear within a few hours after UVB irradiation i.e. apoptosis does not occur within this short period of time. UVB irradiation and reversible permeabilization did not induce the apoptotic shrinking of cells. Based on our measurements in K562 cells UVB irradiation blocked chromatin condensation in the early stage of S phase between 2.2 and 2.4 C values.

In further experiments at lower UVB doses (2, 5, 15 J/m2) chromatin changes were observed only occasioanally, while the weaker UVA irradiation of higher wavelength generated noticable chromatin changes at lower doses. The basic difference between the chromatin structures of exponentially growing K562 cells after gamma and UVB irradiation was that in UVB treated cells there were only a few apoptotic bodies, and chromatin condensation was blocked in its fibrillary stage, preventing the formation of metaphase chromosomes. Examination shed light on similar effects of genotoxic agents such as the local density increase of chromatin or the quite opposite effects involving desintegration, depolarization, exclusion of nuclear material, disruption of nuclear membrane. However, significant differences were also observed, that resulted in a specific chromatin pattern, typical to the agent. Morphological changes generated by genotoxic agents pointed to the possibility that these changes could be categorized. Cellular ethology and viability studies by our long therm scanning (LTS) system after mercuric acetate, led nitrate and nickel chloride confirmed the results of morphological analyses.

These results may contribute to the understanding of the mode of action of genotoxic agents at the chromatin level and contribute to the knowledge of eukaryotic chromatin condensation under normal and pathologic conditions.

NOVEL FINDINGS

By summarizing the morphologic effects on the basis of the similarities and differences between ultraviolet light and gamma irradiation are accentuated:

(1) UV irradiation did not cause significant changes in cellular or nuclear size,

(2) the DNA content expressed in C-value was lower in each synchronized cell population after UV irradiation,

(3) the progression of cell cycle was arrested somewhat earlier in S phase (between 2.2 and 2.4 C), than after gamma irradiation (2.4 C),

(4) UV irradiation blocked chromatin condensation at its fibrillary stage, nuclear structures were blurred and covered with fibrillary chromatin,

(5) although, some apoptotic bodies were seen in mid S phase, they are not typical to UV rather to gamma irradiation,

(6) the lack of metaphase chromosomes indicates that UV damage may release fibrillary chromatin at any stage during chromatin condensation and prevent the folding process. To correlate our results with those of others it was reported that UV can induce G1 or G2 cell cycle arrest in human keratinocytes and neuroblastoma cells, while we have observed that chromatin condensation after UV irradiation was arrested in early S phase between 2.2 and 2.4 C-values. This discrepancy can be resolved by the fact that the C-value of G1 (2.0 C) is close to our observation (2.2–2.4 C). However, the small initial rate of DNA replication indicates that replication has started, thus the C value is probably higher than 2.0 C. The G2 arrest means that chromosomes could not enter metaphase and condensed chromosomes were not formed after UV irradiation. Indeed, we did not see metaphase chromosomes after irradiation, but explain it by the S phase block which is probably maintained throughout the S phase and in G2 phase. Our results are in conformity with the view that UV irradiation generates first giant DNA fragments which could be seen as a fibrillary chromatin cloud. We share the view of Higuchi et al. that the absence of apoptotic cells is an indication of the formation of high molecular weight DNA which precedes ladder-formation of internucleosomal DNA fragmentation associated with apoptosis through caspase activation.

(7) Finally, our data on the genotoxic effects of cadmium, mercury acetate, nickel chloride, lead nitrate, as well as gamma and ultraviolet light irradiations support our notion that preapoptotic events can be categorized by the fingerprinting the injury-specific chromatin changes.

LIST OF ORIGINAL PUBLICATIONS REALTED TO THE DISSERTATION

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3. Trencsenyi, G., **Ujvarosi, K.**, Nagy, G., Banfalvi, G.: Transition from chromatin bodies to linear chromosomes nuclei of murine preB cells synchronized in S phase.

DNA Cell Biol. 26(8):549-556 (2007). Cover page. IF:1,86

4. Banfalvi, G., **Ujvarosi, K.**, Trencsenyi, G., Somogyi, C., Nagy, G., Basnakian, A.G.: Cell culture dependent toxicity and chromatin changes upon cadmium treatment in murine pre-B cells. Apoptosis 12:1219-1228 (2007). IF: 3,04

5. Banfalvi, G., Trencsenyi, G., **Ujvarosi, K.**, Nagy, G., Ombodi, T., Bedei, M., Somogyi, C., Basnakian, A.G.: Supranucleosomal organization of chromatin fibers in nuclei of Drosophila S2 cells. DNA Cell Res. 26, 55-62 (2007). Cover page. IF: 1,86

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