Hyperthermia-induced reorganization of microtubules and microfilaments and cell killing in CHO AA8 cell line

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The aim of this study was to elucidate the effects of hyperthermic treatment on cell morphology and the cytoskeleton in CHO AA8 cell line. The effects of exposure to elevated temperature were analyzed in CHO AA8 cell line by fluorescence microscopy and flow cytometry. The 30min, at 44.5°C heat shock treatment resulted in the collapse of microtubules (MTs) and microfilaments (MFs) around the nucleus followed by their recovery 24h after heating. The initial collapse of these cytoskeletal systems, observed 15min after treatment, was accompanied by the appearance of cells with reduction of volume, shrunken cytoplasm and condensed chromatin. 24h afterwards, there was the increase in the number of cells with restored and extended MT and MF cytoskeletons. Most of them were larger in size compared to the control cells and had multiple nuclei. 48h after heat shock the highest number of the giant cells with alternation in nuclear morphology was seen. Flow cytometry analysis revealed the increase in the number of cells with externalized phosphatidylserine 24h and 48h after hyperthermic treatment. These results suggest that following heat shock, CHO AA8 cells undergo mitotic catastrophe that presumably represents one of the events resulting in apoptosis.

Key words: CHO AA8 cells – actin – tubulin – hyperthermia – cell death

Since it has been proven that hyperthermia above 42 °C has a destructive effect on tumor cells leading to cell death, this kind of treatment has been considered as a promising approach to the therapy of cancer. Additive effects of hyperthemia in combination with chemotherapy or radiotherapy have been demonstrated both in vitro and in vivo [1–11]. There are studies which indicate that cell destruction selectively involves tumors, because of their thermosensitivity caused by low pH and hypoxia whereas the non-malignant cells sustain only minor and reversible damages [3, 12–15]. The changes caused by mild hyperthermia lead to better infiltration and drug absorption into the tumor [14]. It is well known that heat shock destroys enzyme complexes on the cell membrane and interrupt, especially in mitochondria, enzyme system cycles. The thermal stress results not only in changes in stability of cell membrane but alters DNA conformation and gene expression as well. The denaturation of proteins and their subsequent aggregation are believed to be the major changes caused by heat shock, which in turn have a negative impact on various cellular functions leading to hyperthermic cell killing [16–21]. Under these conditions even the cytoskeleton was reorganized leading to changes in cell shape [18, 20, 22–24]. An intact cytoskeleton is essential for maintaining physical integrity of cells and allows them to divide, move themselves and transport intracelullar components [25].The effects of heat shock on cytoskeletal elements differ between different cell lines [24]. Moreover, observation time, the hyperthermic treatment protocol and state of cell spread prior to heat treatment are the additional factors resulting in sometimes contradictory observations [24, 26]

In the present study we wished to elucidate the effects of hyperthermic treatment on cell morphology and the actin and tubulin cytoskeletons in CHO AA8 cell line. Blankenship et al. by treating Chinese hamster ovary cells with lead chromate have confirmed previous reports indicating that apoptosis is one of the cell death modes of CHO AA8 cells [27]. There are other reports showing apoptotic cell death of CHO AA8 cells caused

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by various inducers [28–31]. However, there are very few studies reporting hyperthermia-induced apoptosis in CHO AA8 cells [32, 33]. Apoptosis is a process defined by morphological changes of cells and nuclei such as cell shrinkage, nuclear fragmentation or cleavage, chromatin condensation, and blebbing of the cell membrane [34, 35]. Recent evidence showed the existence of different mechanisms of cell death [35]. One of them is mitotic catastrophe which is demonstrated, among the others, by the presence of the giant multinucleated cells [35–37].

Our work demonstrates that hyperthermic treatment results in selective disruption of microfilaments (MFs) and microtubules (MTs) in CHO AA8 followed by their recovery 24h after exposure to elevated temperature. Moreover, the restoration of the cytoskeletal arrays is accompanied by the appearance of the cells with multiple nuclei that may suggest that one modes of the heat-induced cell death is mitotic catastrophe. Increase in the number of cells with externalized phosphatidylserine 24h and 48h after heating indicate that observed mitotic catastrophe possibly ends in apoptosis.

Material and methods

Cell culture. Chinese hamster ovary cells, CHO AA8 were kindly provided by Prof. M. Zdzienicka (Department of Molecular Cell Genetics, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University, Poland). Cells were maintained in asynchronous growth in monolayer culture with minimum essential medium eagle (MEM; Sigma Aldrich, Poznan, Poland) supplemented with 10% foetal bovine serum (Gibco) and 10ml/L antibiotic-antimycotic stabilized solution (penicillin, streptomycin, amphotericin B; Sigma Aldrich) at 5% CO_2 in humidified 37^0C incubator.

Hyperthermic treatment and cell survival. Cells growing in exponential phase in tissue culture flasks were trypsynized and plated onto glass coverslips in individual wells (at a density of 2 x 10^4cells/well) of six-well plates containing MEM (4ml) with 10% FBS and antibiotics and allowed to attach overnight. The following day, designated as day 1, the plates were sealed with parafilm just before heating and immersed in a 44.5°C water bath for 30min. Immediately after heating, dishes were wiped down and returned to the 37^0C incubator, where they were incubated up to 3 days. Control cells were treated identically except heat shock treatment. Cell viability was assessed by the trypan blue exclusion test. The experiment was repeated 10 times.

Fluorescence labeling. CHO AA8 cells were processed for immunofluorescence microscopy 15min (day 1), 24h (day 2), 48 h (day 3) after heating.

Staining for actin. Cells grown on coverslips were washed briefly with PBS, fixed in 4% paraformaldehyde in HBSS for 15 min, at 37°C and then washed three times with PSB for 5min. The cells were stained for F-actin with phalloidin/TRITC (Sigma Aldrich) in PBS containing 20% methanol for 20 min at room temperature, in the dark. After the labeling cells were rinsed three times in PBS, and mounted in Gelvatol. DAPI (Sigma Aldrich) was used to show nuclei.

Staining for tubulin. Cells on coverslips were prefixed with bifunctional protein crosslinking reagent DTSSP in HBSS [38, 39] for 10 min, at 37°C. Then, the cells were extracted in Tsb containing DTSSP for 10 min, at 37°C; extracted in Tsb for 5 min, at 37°C; fixed in 4% PFA in MTSB; blocked with glicyne and incubated in BSA-TBS two times for 5 min [40]. The cells were stained for tubulin with mouse monoclonal antibody specific for ß-tubulin (Sigma Aldrich) for 45min, at 37°C. This was followed by rinse in PBS-BSA, three times for 5min and incubation with goat anti-mouse IgG-TRITC secondary antibody (Sigma Aldrich) for 45min, at 37°C. Coverslips were rinsed there times in PBS for 5min and mounted in Gelvatol. DAPI (Sigma Aldrich) was used to show nuclei.

The organization of microfilaments, microtubular system and DNA staining was examined with an Eclipse E800 fluorescence microscope (Nikon).

*Flow cytometry.*Apoptosis was analyzed by using the Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen, San Diego, CA). This assay was performed according to the manufacturer's instruction. The fluorescence of 10 000 events per sample was analyzed by FACScan (BD).

Statistical analysis.The nonparametric Mann-Whitney U test (independent group comparison test) was used to determine the difference between experimental points. Results were considered significant at $p<0.05$. The GraphPad Prism software version 4.0 was used for the statistical analysis.

Results

A 30min, 44.5°C heat treatment resulted in similar response among microfilaments and microtubules in the majority of CHO AA8 cells. In majority of cells 15min after hyperthermic treatment, shrunken cytoplasm, reduction of volume, condensed chromatin and complete collapse of microfilaments and microtubules around the nucleus was observed (Fig 1B,C; 2B,C). 24h after heat treatment, recovery of MF and MT cytoskeletons in most cells was seen. The giant, flattened cells showing extended net of stress fibers and bundles of F-actin were also observed. There was the increase in bright F-actin labeling in the center of these cells (Fig 2E). Although the nucleus was unchanged in the proportion of cells with restored cytoskeleton, the nuclear morphology was significantly changed in enlarged, giant forms (Fig 1F; 2F) compared with control ones (showed in miniature at the bottom right corners of Fig 1D; 2D). The nuclei became larger, irregular and multisegmented. The number of cells with micronuclei increased with time and peaked 48h after hyperthermic treatment (Fig 3). Most of the cells observed 48h after treatment recovered or even extended their MF and MT cytoskeleton distribution. However, we also noticed rounded, shrunken cells

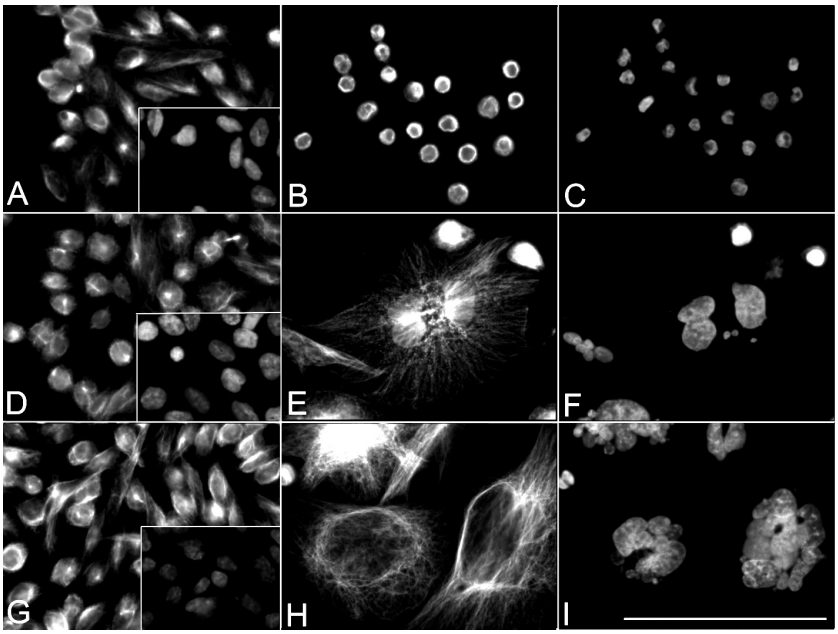

Figure 1. Effects of hyperthermic treatment (30min, 44.5°C) on MT cytoskeleton and nuclei in CHO AA8 cells. The cells were fixed and stained for tubulin with mouse monoclonal antibody specific for ß-tubulin (A, B, D, E, G, H). DAPI was used to show nuclei (C, F, I and in miniature at the bottom right corners of A, D, G). The observations were made: 15min (day 1), 24h (day 2), 48h (day 3) after heat shock treatment (A) 37°C; day 1. (B, C) 30min, 44.5°C; day 1. (D) 37°C; day 2. (E, F) 30min, 44.5°C; day 2. (G) 37°C; day 3. (H, I) 30min, 44.5°C; day 2. Bar = 100µm.

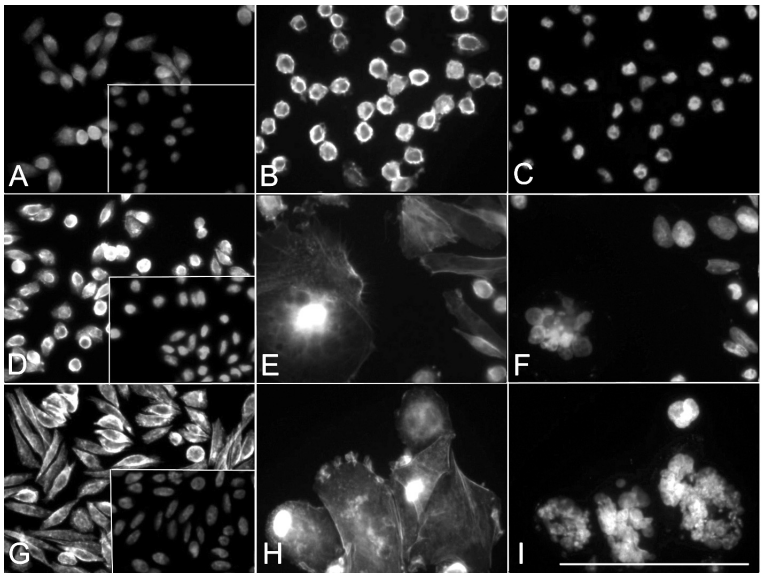

Figure 2. Effects of hyperthermic treatment (30min, 44.5°C) on MF cytoskeleton and nuclei in CHO AA8 cells. The cells were fixed and stained for F-actin with phalloidin/TRITC (A, B, D, E, G, H). DAPI was used to show nuclei (C, F, I and in miniature at the bottom right corners of A, D, G). The observations were made: 15min (day 1), 24h (day 2), 48h (day 3) after heat shock treatment (A,) 37°C; day 1. (B, C) 30min, 44.5°C; day 1. (D) 37°C; day 2. (E, F) 30min, 44.5°C; day 2. (G) 37°C; day 3. (H, I) 30min, 44.5°C; day 2. Bar = 100µm.

with nuclear cleavage and cytoskeleton collapse but without blebs formation (1H; 1I; 2E; 2F).

Cell death was measured by staining cells with PI/Annexin V-FITC. The flow cytometry analysis showed increase in the number of cells exposing phosphatidylserine to the external cellular environment following heat treatment. In comparison to the control cells the number of early apoptotic cells (Annexin V-FITC positive, PI negative) statistically signifi-

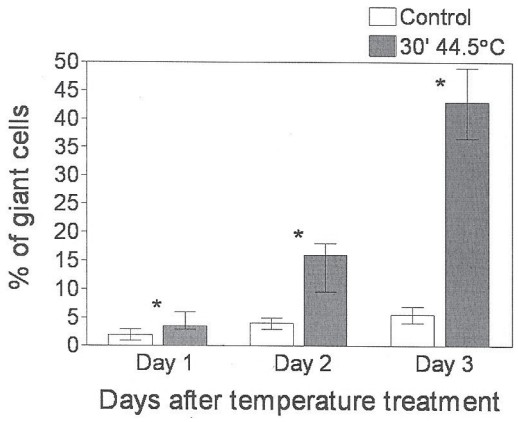

Figure 3. Quantitation of the giant cells (with two or more nuclei) after hyperthermic treatment (30min, 44.5°C). The observations were made: 15min (day 1), 24h (day 2), 48h (day 3) after heat shock treatment. Data are presented as medians and interquartile ranges obtained from five independent experiments based on two random fields with 100 cell counts per field. Mann-Whitney U test was used for statistical analysis. Asterisks indicate statistically significant differences between control and treated samples.

cantly increased at 24h (16.98, median, $p<0.0001$) and 48h (26.04, median, $p<0.0001$) after heat shock treatment (Fig 4B). The number of the late apoptotic (also called secondary necrotic) (Annexin V-FITC and PI positive) cells also increased, compared with control, and was the highest at 48h after treatment (10.38, median, $p<0.0001$) (Fig 4C). In relation to the control cells, the number of necrotic cells statistically significantly increased at 15min (0.31, median, $p<0.0001$) and 48h (1.26, median, $p<0.0001$) following hyperthermia (Fig 4D).

Trypan blue exclusion method revealed that the percentage of necrotic cells was about 2%, what confirmed the above mentioned observation.

Discussion

Our previous studies demonstrated the effects of doxorubicin and UV radiation on the actin cytoskeleton in CHO AA8 cells. We observed a characteristic features of apoptotic rearrangement of the cytoskeletal system, significant relationship between apoptosis and actin reorganization, the

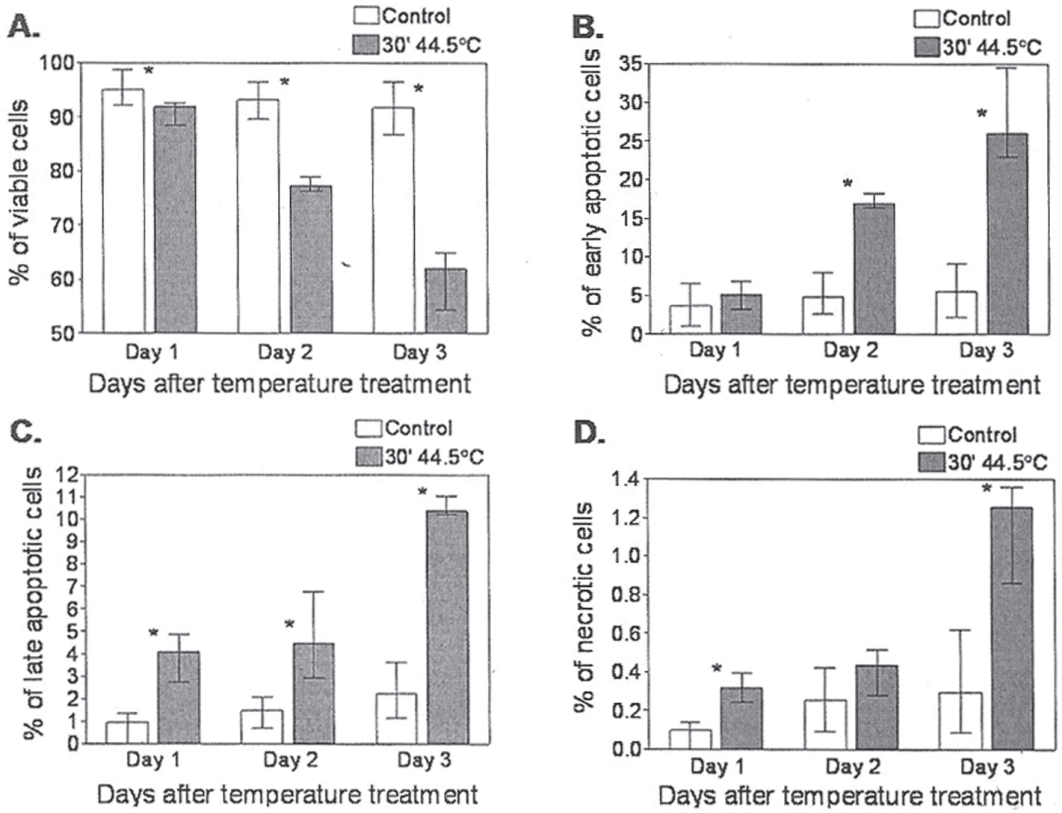

Figure 4. Flow cytometric analysis of viable (A), early apoptotic (B), late apoptotic (C) and necrotic cells (D) in comparison with control cells, after hyperthermic treatment (30min, 44.5°C). The analysis was performed by using flow cytometry with PI and Annexin V-FITC double staining. The observations were made: 15min (day 1), 24h (day 2), 48h (day 3) after heat shock treatment. Data are presented as medians and interquartile ranges obtained from ten independent experiments. Mann-Whitney U test was used for statistical analysis. Asterisks indicate statistically significant differences between control and treated samples.

reduction in the amount of F-actin and its presence in the buds on the cell surface [28, 41]. In the present study, we have shown that the initial response of CHO AA8 cells to heat dose of 30min at 44.5°C is total collapse of MT and MF cytoskeletons. Interestingly, we observed recovery from cytoskeletal disruption 24h after heat treatment. Similarly, Wachsberger and Coss (1990) demonstrated complete collapse of the MF cytoskeletons in synchronous CHO cells and major cytoskeletal disruption of MT arrays following 15min at 45°C heat treatment and total collapse of MT arrays after the 25min, 45°C heat treatment. They observed the restoration of the cytoskeletal arrays in the portion of the population 20h following heat treatment [26]. Recovery from heat-induced cytoskeletal alternation was also reported by the others [24].

As it was mentioned in Results, the first response of the cell population to the heat shock treatment was the reduction of volume, chromatin condensation and cytoskeletal disruption. It was followed by the recovery of the MT and MF arrays, the appearance of flattened cells with increase in size and multiple nuclei and the increase in the number of cells with externalized phosphatidylserine. It was previously indicated that there are at least two mechanisms of cell death following heat treatment, the rapid mode and the slow mode of cell death. The latter was suggested to occur after the cell recovering from inhibition of macromolecular synthesis and was followed by division abnormalities resulted in the appearance of the cells with multiple nuclei [42]. Nakahata et al., suggested that the organelles targeted as the 'victims' of heat shock were centrosomes and implied that the lethality of cells was connected with mitotic catastrophe [43]. These findings were subsequently confirmed by others who observed that the exposure of cells to heat stress resulted in shock-affected centrosomes and variety of mitotic abnormalities [44–47]. There are a few distinctive characteristics of mitotic death. First of all, it appears in p53 non-functional and p53-mutated tumors, generally in the cells lacking wild-type p53 function, thus, having a survival potential. The G1/S checkpoint is absent in those cells as well as interphase apoptosis which is coupled to this checkpoint. This type of cell death is also associated with aberrant mitosis, formation of giant cells (containing two nuclei or many micronuclei) and delayed apoptosis. Surprisingly, mitotic death is also correlated with such phenomena as restitution, micronucleation and polyploidy, not associated with mitosis [35, 36]. Marked increase in cell size and the appearance of cells with multiple nuclei observed in this study might suggest that the heat shock affected those cells possibly at centrosomes leading to divisions errors and, eventually, to mitotic catastrophe. This theory would be in agreement with the previous findings indicating the existence of some kind of relationship between hyperthermia and mitotic death [43]. Hut et al. suggested that, although hyperthermia does not belong to genotoxic stressors, and, by itself, does not cause DNA damages, it may be responsible for the genome instability possibly through inducing centrosome damage. Induced by thermal stress, centrosomal protein damage leading to multipolar spindles formation may cause severe errors in cell division [44] and possibly result in mitotic catastrophe. It is also worth noting that used in this report, CHO AA8 cell line presumably have the Thr^{211}-to-Lys^{211} change and, as the result of this single mutation, loss of G1 phase checkpoint executed by p53. Conversely, G2 and S checkpoints were not changed [48]. In our study, results obtained from flow cytometry analysis revealed that the number of cells with externalized phosphatidylserine was the highest at 48h after heat treatment what corresponds with increased number of giant cells and cells with some apoptotic features. Results obtained by others suggest, however, that those observation can not help us distinguish between the types of hyperthermic-induced cell death, because both the cells undergoing apoptosis and mitotic catastrophe externalize phosphatidylserine in the plasma membrane [49]. It lets us to assume that hyperthermia induces mitotic catastrophe and apoptosis in CHO AA8 cells but without quantitative discrimination between these cases. It may also suggest that mitotic catastrophe ends in apoptosis. To assess it, further investigation is required.

In conclusion, our data show that hyperthermia is a method that may cause severe alterations in the actin and tubulin arrays of CHO AA8 cells followed by the recovery of these cytoskeletal arrays in a portion of damaged cells. We suggest that one of the modes of CHO AA8 cells death is mitotic catastrophe which probably ends in apoptosis. We believe that investigations of the involvement of actin and tubulin systems in the process of cell death under different conditions may provide a new insight into the issue concerning cancer therapy. Considering the growing amount of tumor- targeting drugs, it is important to find within the principal pathway of the malignant cells death and the treatment with minimum side effects.

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