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The role of intracellular zinc in modulation of life and death of Hep-2 cells

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Abstract

Varying intracellular concentrations of zinc in laryngeal Hep-2 cells in relation to changing cultivation conditions *in vitro* were determined by atomic absorption spectrophotometry. Upon standard cultivation in DMEM with 10% serum, the mean concentration of zinc was determined at 0.88 ± 0.09 μg/mg protein, with substantially decreased values in the cells exposed to a low-serum medium. Next, the study of the effects of a series of physiological and supraphysiological concentrations of $ZnSO_4$ on laryngeal cells and their correlation with determined intracellular concentrations of zinc was performed. It was found that zinc concentrations above 100 μM were toxic to Hep-2 cells, inducing cell death in the interval of 96 h as determined by videomicroscopy, selective nuclear staining, and immunofluorescence detection of caspase-3 and specific cytokeratin 18 fragment. Both types of cell death were observed, with apoptosis being induced at moderately toxic zinc concentration of 150 μM and necrosis at higher zinc concentrations of 300 μM and 750 μM, respectively. Lower concentrations (1.5–100 μM), on the other hand, did not produce any measurable changes in cell morphology and function in the same time interval. Zinc at concentration of 1.5 μM was found to slightly enhance proliferation of Hep-2 cells up to the certain time point, which seemed to correlate with maximal tolerable momentary intracellular level of zinc. These results illustrate the importance of determining the intracellular levels of zinc when trying to characterize the effect of exogenous zinc on life and death of laryngeal cells.

Introduction

Zinc is an important biogenic element whose availability is considered critical for normal functioning and proliferation of cells (Chai *et al.* 1999). Zinc is required for enzymatic catalysis, gene expression, DNA synthesis and many other specialized functions, such as tissue regeneration, neurotransmission and photoreception (Smart 1992; Vallee & Falchuk 1993). Moreover, zinc is a powerful guardian of cells as it can protect them against oxidative stress, mainly by acting on superoxide dismutase and catalase (Truong Tran *et al.* 2001).

Many studies have shown that zinc is also implicated in modulation of apoptosis, programmed cell death. It has been demonstrated in several cell lines that zinc can prevent apoptosis induced by different factors, and that cells maintained under conditions of zinc deficiency (induced by either low-zinc environment or zinc chelators) can undergo apoptosis spontaneously (Hyun *et al.* 2000; McCabe *et al.* 1993; Szuster-Ciesielska *et al.* 2000). It is postulated that zinc ions suppress DNA fragmentation, one of the characteristic features of apoptosis, by inhibiting the calcium/magnesium-dependent endonuclease (Lohmann & Beyersmann 1993; Wolf *et al.* 1997). Furthermore, it has been reported recently that zinc acts even further upstream, blocking caspase-3, an execution cell death protease (Hyun *et al.* 2000; Chai *et al.* 1999; Chimienti *et al.* 2001). This protease is often found activated in apoptotic cells, leading to the cleavage of crucial cellular proteins, i.e., poly (ADP-ribose) polymerase and fodrin, which are responsible

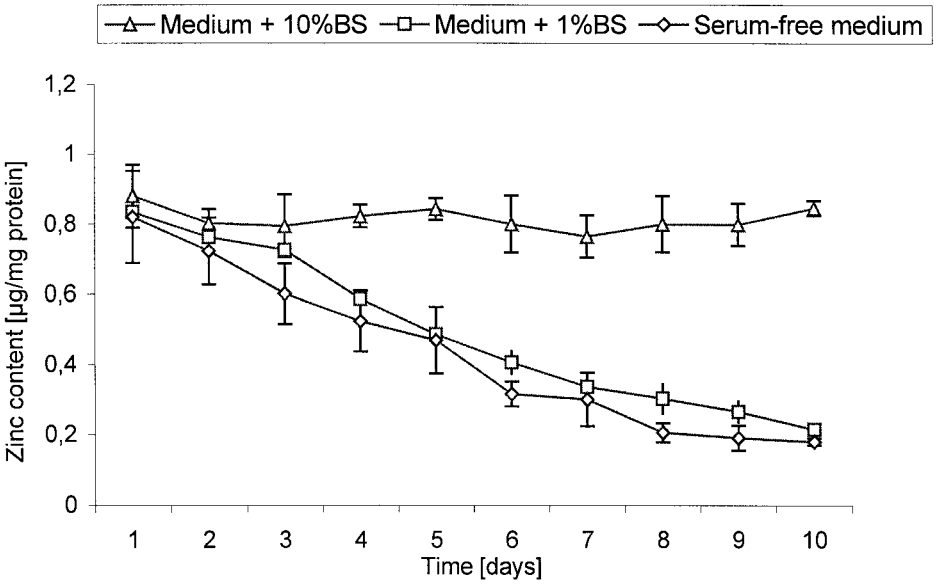

Figure 1. Changes in concentration of intracellular zinc in Hep-2 cells cultivated for 10 days in DMEM with varying serum content. Values represent the mean ± S.D. of three independent measurements.

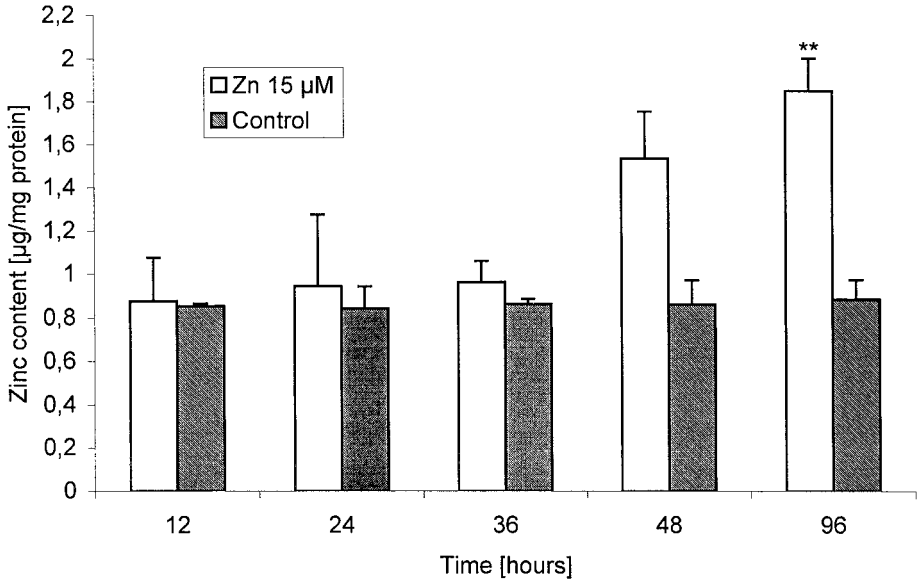

Figure 2. Changes in concentration of intracellular zinc in Hep-2 cells cultivated in DMEM with 10% BS and supplemented with zinc sulfate at 15 μM during 96 h. Data represent the mean ± S.D. of three independent experiments. **: $P < 0.01$.

for the entry of the cell into apoptotic process (Aiuchi *et al.* 1998; Truong Tran *et al.* 2001).

Evidence exists, however, that zinc is capable of inducing apoptosis at physiological concentrations, and that even higher concentrations can produce irreversible changes in exposed cells leading to toxic cell death. Therefore, it seems that different concentrations of zinc may have disparate effects ranging from stimulation to inhibition of cell growth or cell death, depending upon the cell type and some other external factors and conditions (Cario *et al.* 2000; Fraker & Telford 1997; Liang *et al.* 1999; Provinciali *et al.* 1995).

Human larynx is an organ exposed to a wide variety of chemicals from the external environment (Catimel 1996; Lewis 1991). Thus laryngeal cells

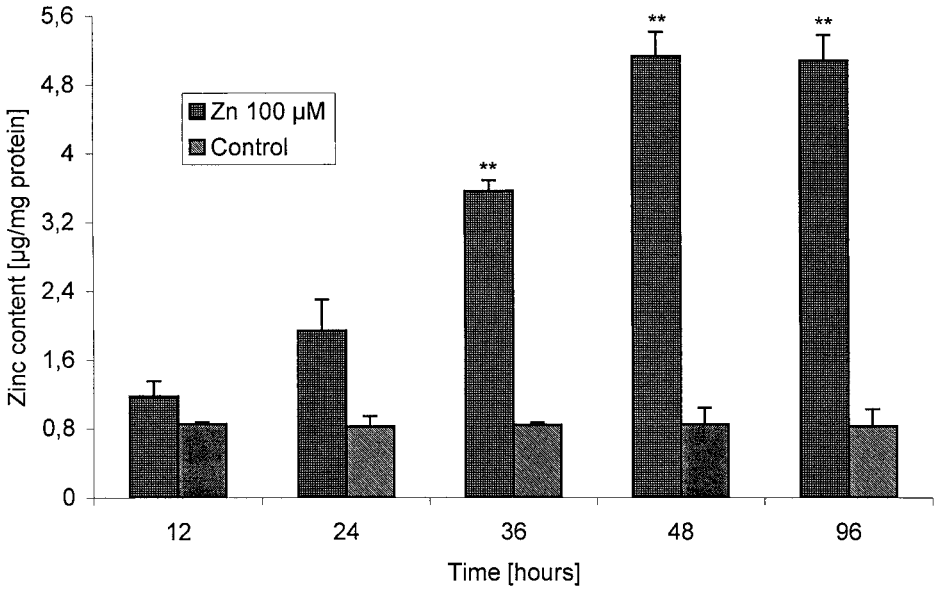

Figure 3. Changes in concentration of intracellular zinc in Hep-2 cells cultivated in DMEM with 10% BS and supplemented with zinc sulfate at 100 μM during 96 h. Data represent the mean $\pm$ S.D. of three independent experiments. **: $P < 0.01$.

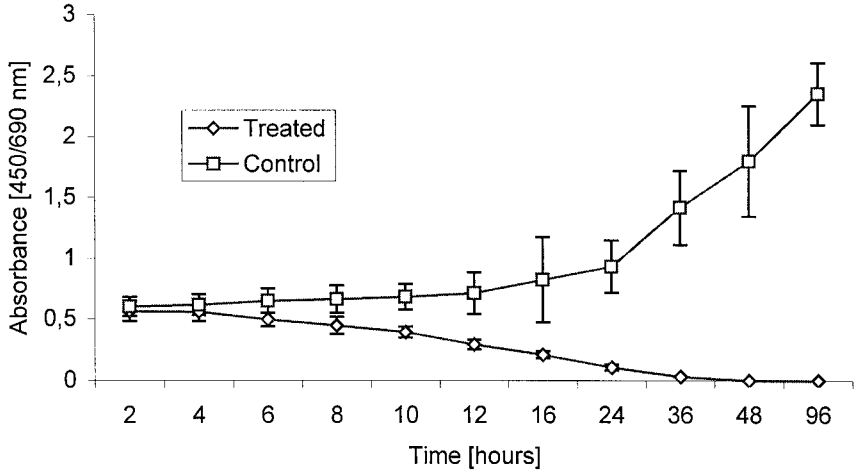

Figure 4. The influence of zinc sulfate at concentration of 300 μM on metabolic activity of Hep-2 cells during 96 h. Data represent the mean $\pm$ S.D. of three independent experiments.

are frequently overstressed and their growth as well as function may undergo potentially dangerous oscillations, with this situation likely resulting in the appearance of some clinical symptoms, such as inflammation or cancer (Szyfter *et al.* 1999). Due to the fact that zinc is known to protect cells and enhance their regenerative abilities, it might be of interest to find out if there is any association between the intracellular concentrations of zinc in larynx and the normal function of laryngeal cells. To our knowledge, there are limited experimental data available concerning this subject. Thus, the aim of this study was to determine the intracellular concentrations of zinc in Hep-2 cells, a human epitheloid cell line derived from carcinoma of larynx, upon different cultivation conditions. Our second goal was to assess the effects of zinc on proliferation, morphology and selected biochemical parameters in the above-mentioned cells and correlate them with the determined intracellular concentrations of zinc. Finally, while using a wide range of concentrations including physiological as well as supraphysiological, we have attempted to character-

Table 1. Zinc concentration in cultivation media with different serum content.

Sample	Concentration of zinc in μM
DMEM without serum	0.29 ± 0.02
DMEM with 1% BS*	0.65 ± 0.08
DMEM with 5% BS	1.08 ± 0.10
DMEM with 10% BS	2.10 ± 0.04

DMEM* – Dulbecco's modified Eagle's medium.
BS** – bovine serum.

ize potentially harmful as well as beneficial effects of zinc on Hep-2 cells in relation to its intracellular concentrations.

Materials and methods

Cell line

Human laryngeal cell line Hep-2 (ECACC, No. 86030501, Porton Down, United Kingdom) was grown in Dulbecco's modified Eagle's medium (Sevapharma), supplemented with 10% bovine serum (Bioveta), 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were maintained in plastic tissue-culture dishes (Nunclon) in an incubator with 5% CO_2 at 37 °C.

Chemicals

Zinc sulfate ($ZnSO_4$. 7 H_2O) (Sigma-Aldrich) was dissolved in a serum-free medium, sterilized by ultrafiltration, and stored as a stock solution of 1 M in a refrigerator until use. Cells were treated with a final concentration of 1.5, 15, 100, 150, 300 and 750 μM $ZnSO_4$. 7 H_2O for up to 96 h.

WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) was purchased from Boehringer Mannheim-Roche, DAPI (4′, 6-diamidino-2-phenylindole) and Triton-X were obtained from Sigma-Aldrich. Monospecific antiserum for the detection of caspase-3 was obtained from New England Biolabs, Inc. and monoclonal antibody M30 was from Boehringer Mannheim-Roche. Secondary antiodies were from Molecular Probes, Inc. All other chemicals were of analytical grade if not stated otherwise.

Zinc measurement

Harvested cells were rinsed three times with cold PBS, with each rinsing followed by centrifugation for 5 min at 1500 rpmi (JOUAN MR 22, rotor SWM 180.5) at room temperature (RT). Next, the cells were mineralized, atomized, and assayed for zinc by atomic absorption with an inductively coupled plasma emission spectrometer MSD 5972 (Hewlett Packard). Aliquots of cell samples prior to analysis were assayed for protein as reported elsewhere (Bradford 1976). Zinc values are given as μg zinc/mg protein. The same procedure was used for measurement of zinc in medium (zinc values are given in μM).

WST-1 assay

Hep-2 cells (6,000 cells/well) in 200 μl of DMEM containing 10% bovine serum were seeded in four 96-well microtiter plates, with the first two columns of wells without cells (blank). The cells were incubated 24 h at 37 °C and in 5% CO_2. After incubation, the medium was replaced with a medium containing a tested concentration of zinc sulfate and cultivated for different time intervals at 37 °C and 5% CO_2. After each time interval, 100 μl of WST-1 was added. The cells were further incubated for 2 h. The absorbance was recorded at 450 nm with 650 nm of reference wavelength by a scanning multiwell spectrophotometer Titertek Multiscan MCC/340. In all cases, the absorbance of the tested substance in medium alone was recorded to determine whether it interfered with the assay. Each tested solution was tested in sixteen independent spots. Experiments were done in triplicate.

Time-lapse videomicroscopy

Hep-2 cells were seeded into a plastic tissue-culture dish and left for 24 h in an incubator with 5% CO_2 at 37 °C. Next day the standard medium was replaced with a medium containing different concentrations of zinc sulfate. The tissue-culture dish was transferred into a 37 °C-heated chamber where all recordings were performed. Cells were observed continuously over the 96 h period, using an inverted microscope (Olympus IMT-2) equipped with a long-working-distance condenser, and a 20 × phase contrast lens. For time-lapse recording, the microscope was equipped with a Mitsubishi CCD-100E camera and connected to a Mitsubishi video recorder HS-S5600. The recording was performed in a 480 mode, with a slowing factor

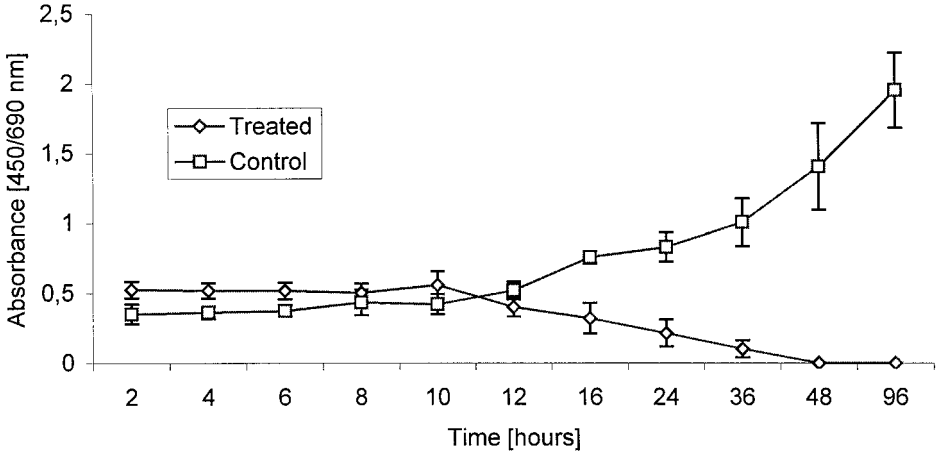

Figure 5. The influence of zinc sulfate at concentration of 150 μM on metabolic activity of Hep-2 cells during 96 h. Data represent the mean ± S.D. of three independent experiments.

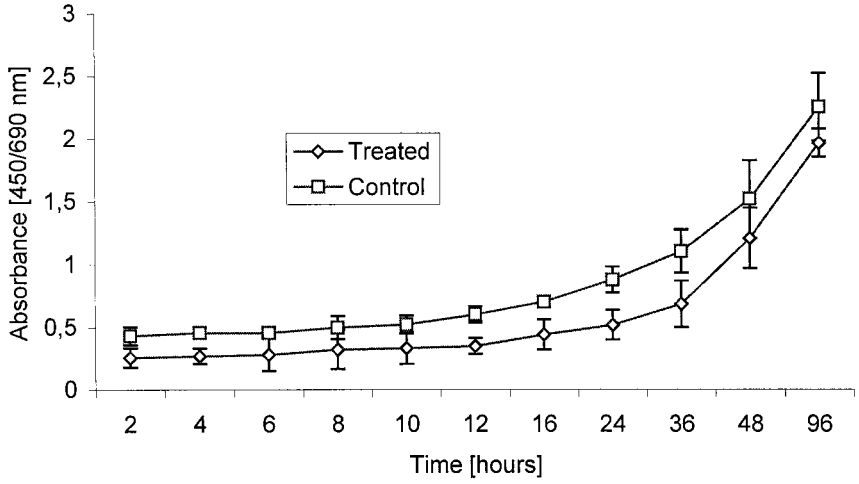

Figure 6. The influence of zinc sulfate at concentration of 100 μM on metabolic activity of Hep-2 cells during 96 h. Data represent the mean ± S.D. of three independent experiments.

of 160 and it continued for 96 h, with a subsequent video analysis. The chosen sequences were converted to digitalized format, processed by the software Adobe Premiere 6.0, and analyzed.

Fluorescence microscopy

Nuclear staining with DAPI. Hep-2 cells were seeded into modified cytospin chambers and allowed to grow in an incubator with 5% CO_2 at 37 °C for 24 h. After 24 h, the standard medium was exchanged for a medium with tested concentrations of zinc sulfate and cells were treated for different time intervals. After the treatment, the cells in cytospin chambers were centrifuged for 5 min at 500 rpmi (JOUAN MR 22, rotor SWM 180.5), washed with cold PBS and air-dried. The cells were then labeled with DAPI and covered by a coverslip with mounting fluid. The cells were examined under a fluorescence microscope Nikon Eclipse E 400 (excitation filter 330–380 nm and emission filter 420 nm) equipped with a digital color matrix camera Basler A113CP**.** Photographs were taken using the software LUCIA DI Image Analysis System LIM and analyzed. Experiments were done in triplicate.

Immunocytochemical detection of caspase-3 and the specific cytokeratin 18 fragment. Hep-2 cells were treated as described in the previous section. After the treatment, the cells in cytospin chambers were centrifuged for 5 min at 500 rpmi (JOUAN MR 22, rotor SWM 180.5) at RT, the medium was carefully

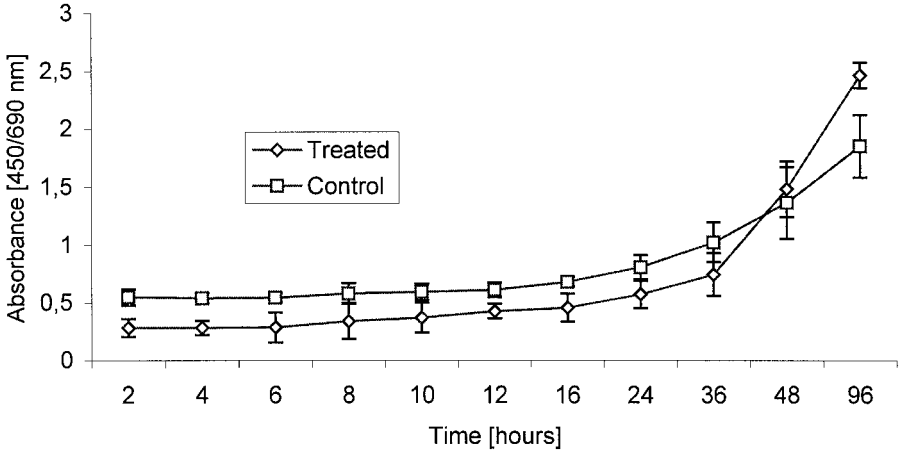

Figure 7. The influence of zinc sulfate at concentration of 15 μM on metabolic activity of Hep-2 cells during 96 h. Data represent the mean ± S.D. of three independent experiments.

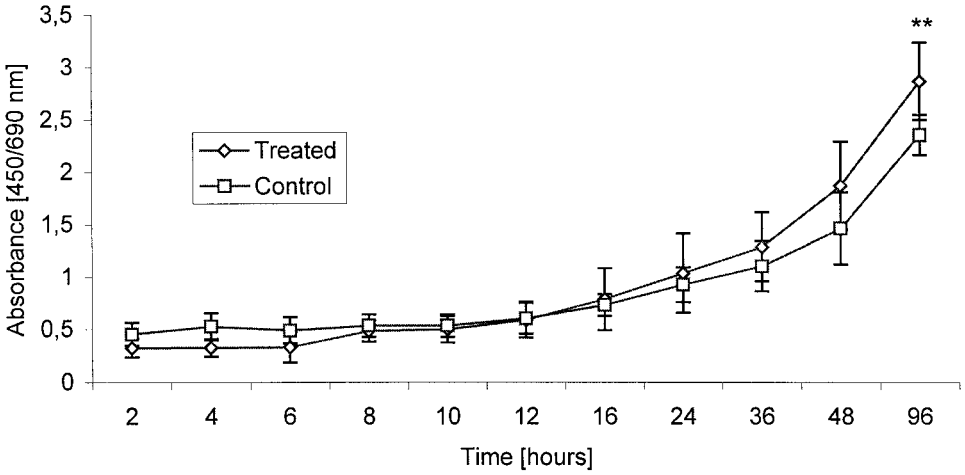

Figure 8. The influence of zinc sulfate at concentration of 1.5 μM on metabolic activity of Hep-2 cells during 96 h. Data represent the mean ± S.D. of three independent experiments.
**: $P < 0.01$.

aspirated and the cells were fixed with 1 ml of 4% formaldehyde. After second centrifugation (conditions see above), cytospin chambers were disassembled and the cells on a slide were air-dried. Detection of an activated (cleaved) form of caspase-3 was performed using monospecific antiserum. Activated caspase-3 is responsible for specific fragmentation of the cytokeratin 18 (CK18). We monitored this process by immunocytochemical detection of the specific caspase-cleaved epitope of the CK18 by monoclonal antibody M30. Various modifications of immunofluorescence techniques were used for visualization. Briefly, the slides with cells were rinsed three times with phosphate saline buffer with Triton-X (PBS-T) and then treated to skimmed milk for 30 min at RT. After this period, a primary antibody was added to the cells and the slides were left overnight in a cultivation chamber MIST at 4 °C. The slides were then rinsed three times with PBS-T buffer, a secondary antibody was added, and the cells were incubated in the cultivation chamber MIST for 90 min at RT. After incubation, the cells were rinsed three times with PBS-T buffer and once in distilled water. The specimens were optionally post-labeled with DAPI, mounted into a special anti-bleaching medium and examined under a fluorescence microscope Nikon Eclipse E 400 equipped with the digital color matrix camera Basler A113CP. Photographs were taken using the software LUCIA DI Image Analysis System LIM and analyzed. In all experiments, the system of immunological control was

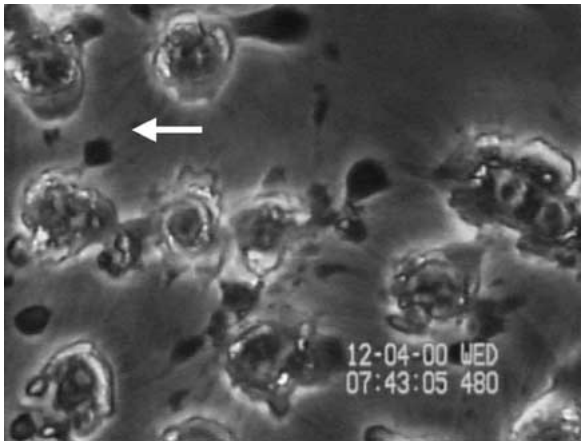

Figure 9. Morphological appearance and behavior of Hep-2 cells after treatment with 750 μM zinc sulfate during 2 h. The shrinking cells leave their anchor fibers adherent and release small membrane fragments (arrow). The image represents the printout of digitalized time-lapse sequences.

employed to avoid false positive or negative staining reactions. Experiments were done in triplicate.

Statistics

Statistical analysis was carried out with a statistical program GraphPad Prism. We used one-way Anova test with the posttest Dunnet's. Results were compared with control samples, and means were considered significant if $P < 0.01$.

Results

Cellular zinc concentration

The determined concentrations of zinc in the employed cultivation medium with or without bovine serum are shown in Table 1. The standard cultivation medium DMEM with 10% bovine serum contained zinc at 2.10 $\pm$ 0.04 μM, and this concentration decreased with reduced serum content, being at 0.29 $\pm$ 0.02 μM in serum-free medium. Hep-2 cells, which were standartly cultivated in DMEM supplemented with 10% bovine serum, contained zinc at 0.88 $\pm$ 0.09 μg/mg protein. When exposed to the medium with low serum content or without serum, the intracellular concentration of zinc dropped during 10 days cultivation to about
0.21 $\pm$ 0.1 and 0.18 $\pm$ 0.08 μg/mg protein, respectively. The changes in the intracellular zinc levels in Hep-2 cells during cultivation in media with different serum content are shown in Figure 1.

Supplementation of the standard cultivation medium with 1.5 μM zinc sulfate resulted in no significant increase in the intracellular zinc levels over the time period of 96 h. Zinc uptake was significantly enhanced with employed higher extracellular zinc doses (15 and 100 μM) during the same time period as shown in Figures 2 and 3.

Zinc toxicity

This colorimetric assay is based on the cleavage of tetrazolium salt by mitochondrial succinate dehydrogenase in viable cells. It was used to assess the effect of zinc at different concentrations on viability and proliferation of Hep-2 cells in the time interval of up to 96 h. Zinc sulfate at the concentrations of 750 and 300 μM proved to be significantly toxic over a 24 h period (Figure 4). The concentration of 150 μM did not decrease Hep-2 cells viability during 12 hours of the treatment; however, in the longer time course (starting with 16 hours after the beginning of treatment) its toxicity was quite apparent (Figure 5). Neither viability nor proliferation of Hep-2 cells were changed over a 96 h period when treated with zinc sulfate at the concentration range 100–15 μM (Figures 6 and 7). A slight proliferation enhancement was observed in cells treated with 1.5 μM zinc sulfate (Figure 8).

Dynamic morphology of cells treated with zinc

Morphology of epitheloid cells exposed to the highest employed zinc dose (750 μM) showed marked changes as soon as 0.5 h after the beginning of the treatment. The cells were losing their adherence, followed by a typical cytoskeletal collapse, cytoplasmic coarsening and formation of microevaginations. The entire process ended up by a chaotic cell shrinkage and fragmentation with general release of cell content. After 2 h, there were no intact cells in the exposed culture (Figure 9).

Compared to the highest employed concentration, which induced immediate changes resulting in toxic cell death, the concentration of 300 μM produced much more discrete changes in the cells during first 4 h. Still, longer periods of continuous treatment (5–10 h) have resulted in gradual structural as well as functional cell damage, with the appearance in some cells of blebs followed by cytoskeletal collapse and cell shrinkage (Figure 10).

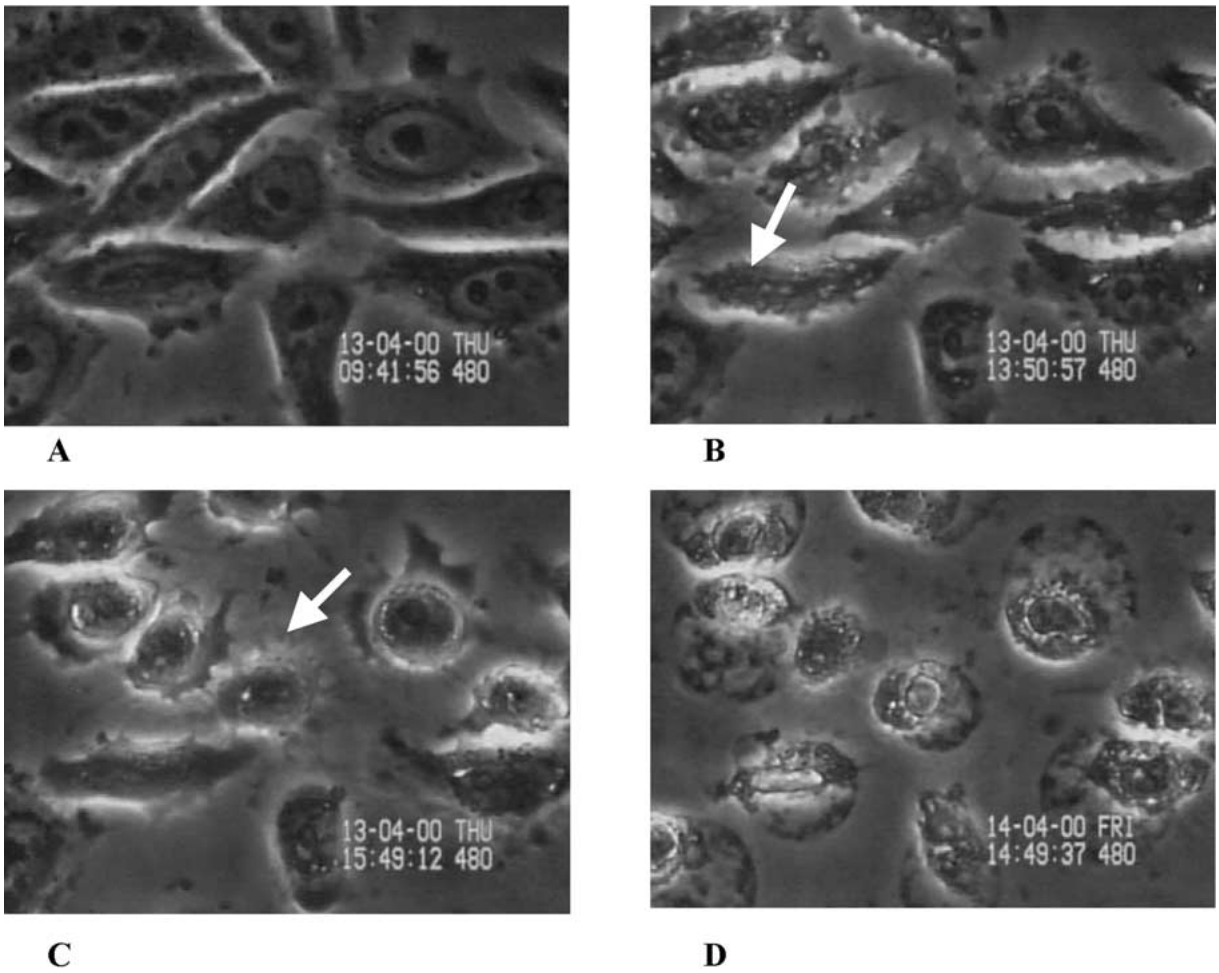

Figure 10. Morphological appearance and behavior of Hep-2 cells after treatment with 300 μM zinc sulfate during 24 h. Until 4 h of the treatment, the cells did not show any pathological changes (panel A). During the time period of 5–10 h, in some cells were visible blebs (arrow – panel B), and gradually their cytoskeleton underwent irreversible changes (arrow – panel C). After 24 h, there were no intact cells (panel D).

When treated with 150 μM zinc sulfate, Hep-2 cells at first seemed to behave and proliferate in a normal way as compared with control cell cultures, but at 36 h after the beginning of the treatment the blebbing affecting gradually all cells as well as other typical features of cell death could be observed (Figure 11).

Concentrations of zinc sulfate below 150 μM (i.e., 100, 15 and 1.5 μM) were not found toxic or otherwise affecting cell morphology and behavior during 96 h (Figure 12).

Fluorescence visualization of Hep-2 cell structures after zinc treatment

Nuclear staining with DAPI

The selective staining of treated nuclei with DAPI revealed significant nuclear changes, such as chromatin dispersion and fragmentation (Figure 13), occurring after the highest employed zinc concentrations (750 and 300 μM). Lower concentrations, in particular 150 μM, have had ambiguous effect on nuclei, producing in some cells after 24 h lasting treatment observable chromatin fragmentation as well as non-specific disintegration (Figure 14). No changes in nucleus morphology were observed after the treatment with zinc concentrations below 150 μM during 96 h.

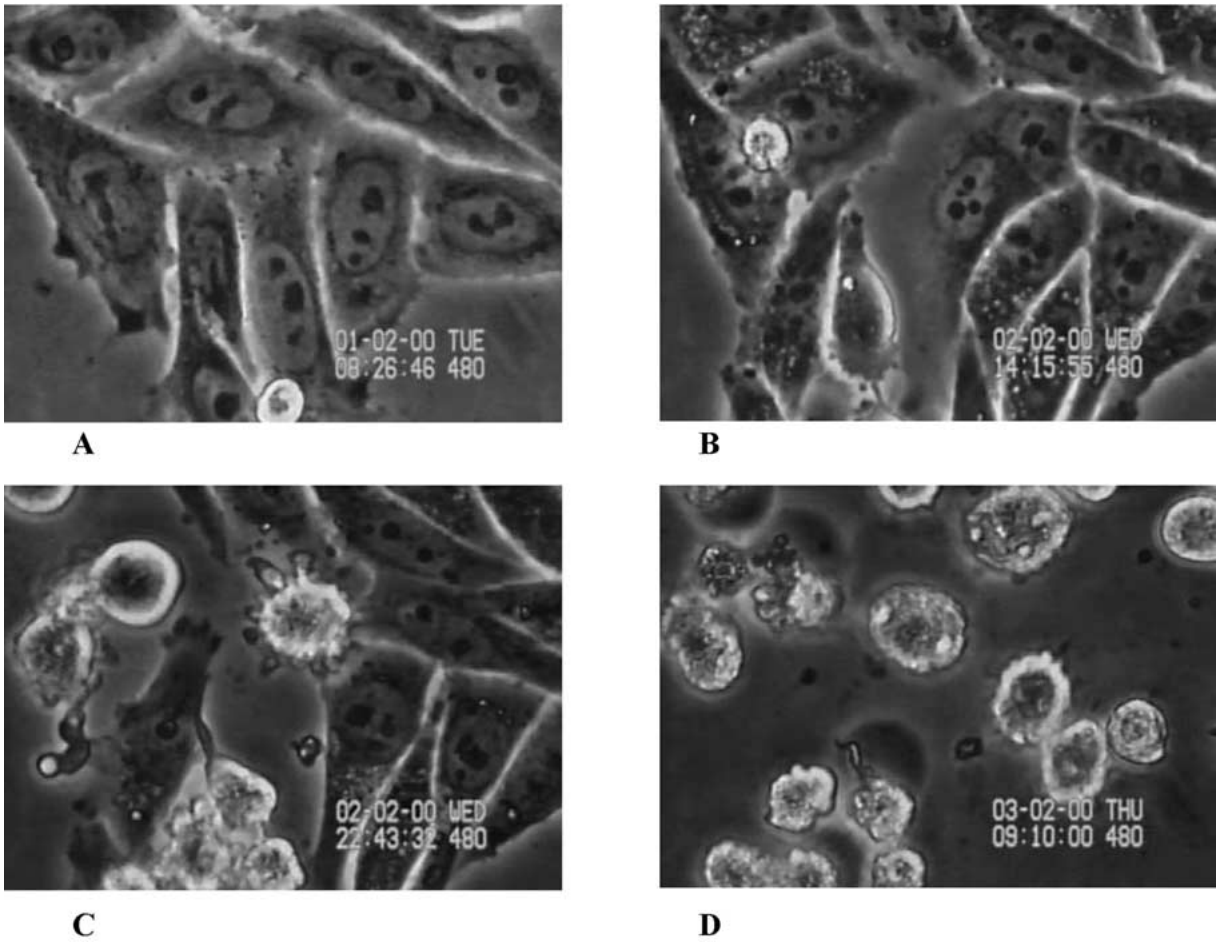

Figure 11. Morphological appearance and behavior of Hep-2 cells after treatment with 150 μM zinc sulfate during 48 h. There were no marked changes in cell morphology and behavior during first 36 h of the treatment (panels A and B). After 36 h (panel C), all the cells asynchronously began blebbing, followed later by other marks of cell death (cell shrinkage and secondary necrosis – panel D). The images represent the printouts of digitalized time-lapse sequences.

Immunocytochemical detection of caspase – 3 and the specific cytokeratin 18 fragment

The detected levels of the activated form of caspase-3 and its specific product CK18 were not found to be significantly different in the cells treated with 750 and 300 μM zinc sulfate and in the control culture during 24 h (Figure 15). Zinc sulfate at concentration of 150 μM increased the levels of both activated caspase-3 and its fragment in the treated cells in a 24 h interval; however, during subsequent treatment its effect became less pronounced as shown in Figure 16.

Discussion

It has been shown in numerous studies that the intracellular concentration of zinc and its dynamic changes are of critical importance in many cell types, influencing their long-term growth, physiological performance and survival (Fraker & Telford 1997). This is particularly true for epithelial tissues of the respiratory system, which act not only as a potent barrier against various environmental particles but also produce scores of useful chemicals (Truong Tran *et al.* 2001).

In our study, we have attempted to determine the intracellular levels of zinc in laryngeal epitheloid Hep-2 cells and to study the influence of zinc deprivation as well as zinc supplementation on their growth and survival. Upon standard cultivation conditions, Hep-2 cells maintain relatively stable intracellular zinc concentrations (see Figure 1) which are comparable with reported concentrations in other studied cells (Szuster-

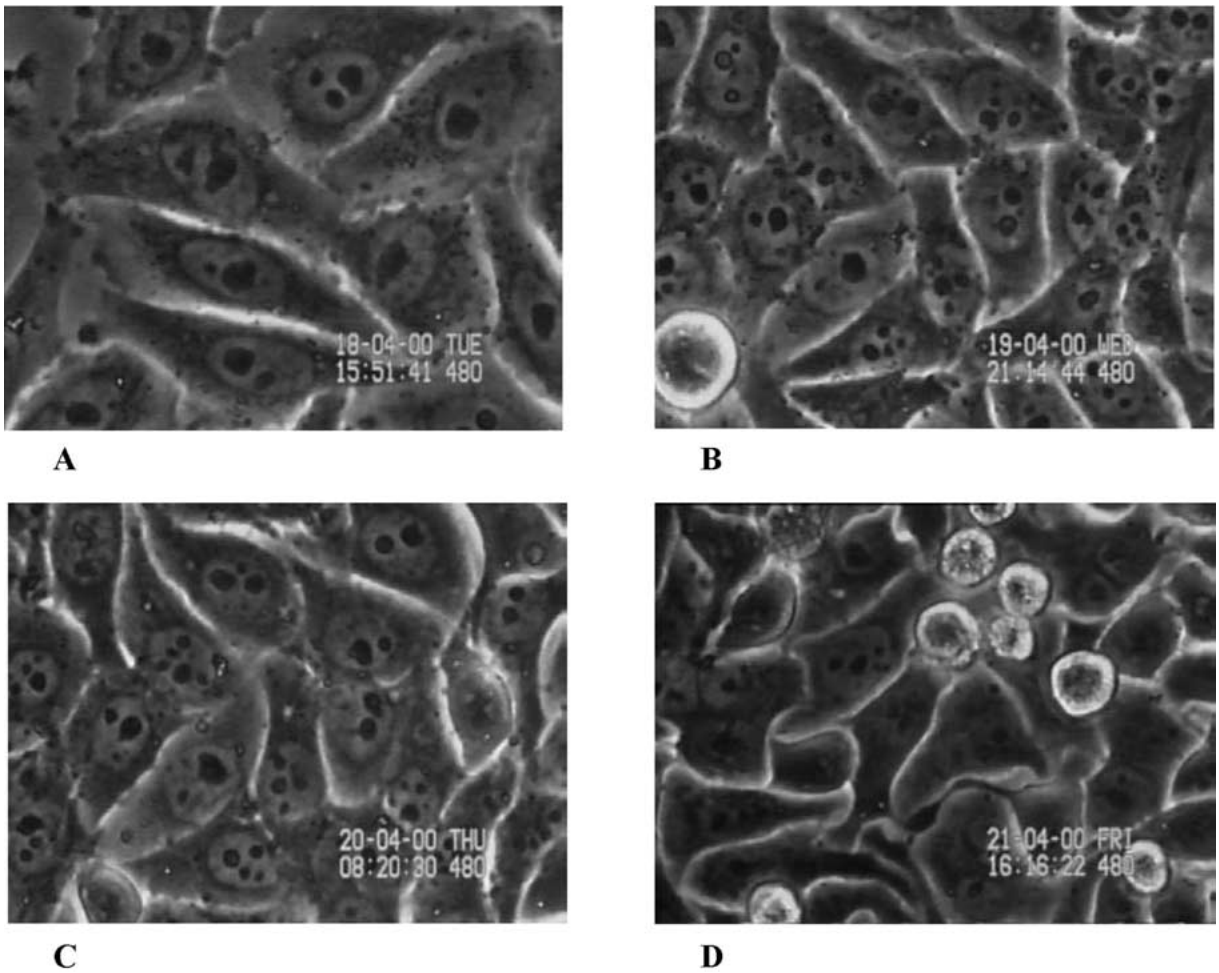

Figure 12. Morphological appearance and behavior of Hep-2 cells after treatment with 100 μM zinc sulfate during 96 h. A continuous increase in the cell number was observed during the treatment time (panel A – D), with no detectable differences in comparison with controls. After 96 h (panel D), full confluence of the test system was reached and the cells began to shrink. The same results were achieved with 15 and 1.5 μM zinc sulfate (observations not shown). The images represent the printouts of digitalized time-lapse sequences.

Ciesielska *et al.* 2000). In addition, our findings imply that human larynx as much as some other target organs (prostate gland) (Liang *et al.* 1999) accumulates significant quantities of this biogenic element. The observed time and concentration dependent intracellular zinc increase in Hep-2 cells correlates with the findings recorded in C6 rat glioma cells. These cells have been shown to significantly internalize zinc ions only upon external zinc concentration exceeding the established threshold of 200 μM. The authors proved that beyond-threshold concentrations markedly increase the intracellular zinc levels, but this process occurs on the expense of cell viability maintenance, most probably due to the zinc induced damage to the cell membranes. On the other hand, below-threshold external zinc concentrations had no effect on the zinc uptake in glioma cells and this was attributed to a regulatory system protecting these cells against high intracellular concentrations of this metal (Haase & Beyersmann 1999). Considering these results it would be interesting to see whether this protective system is efficient in glioma cells even during longer time intervals than 3 h as our own results indicate that Hep-2 cells slowly increased intracellular zinc concentrations upon their treatment with beyond-threshold concentrations during 96 h (Figures 2 and 3).

To find out to what extent intracellular concentrations change when the external zinc sources are diminished or depleted, we exposed Hep-2 cells to a low-serum or serum-free environment during 10 days.

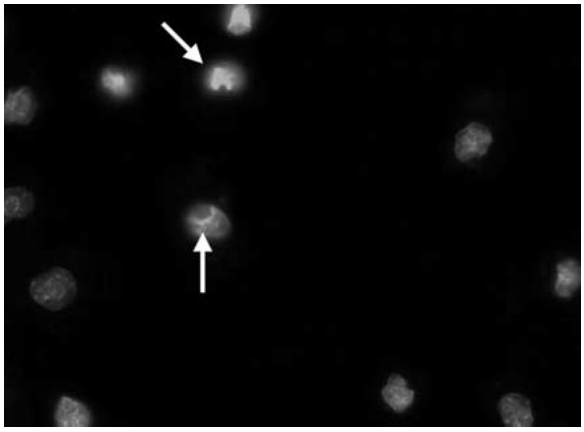

Figure 13. Morphology of the Hep-2 cells nuclei exposed to treatment 750 μM zinc sulfate during 2 h. The extensive chromatin disintegration and fragmentation are visible (arrows).

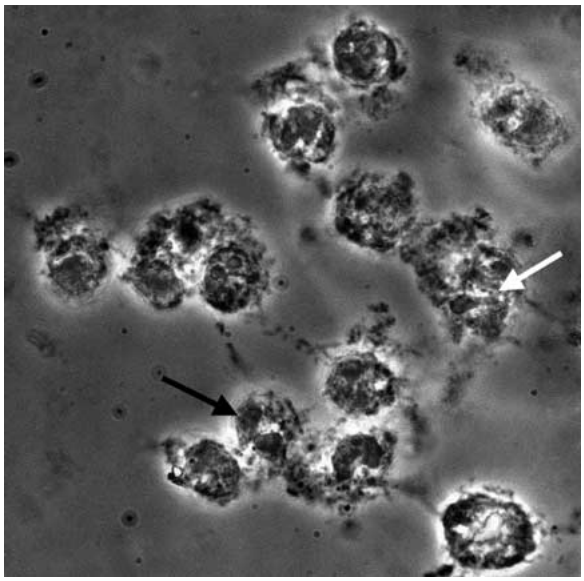

Figure 14. Morphology of the Hep-2 cells nuclei exposed to 150 μM zinc sulfate during 24 h. Specific chromatin fragmentation characteristic of apoptosis (black arrow) as well as typical necrotic dispersion (white arrow) are clearly visible. The presented photograph has arisen from the superimposed phase contrast and fluorescence images.

We have chosen this model over the often used zinc-specific chelators (Ahn *et al.* 1998; Hyun *et al.* 2000) mainly due to two reasons. Firstly, zinc chelators are very potent, depriving cells of the entire zinc content within hours (Chimienti *et al.* 2001; Marini & Musiani 1998). Therefore, it results difficult to study potential adaptation mechanisms the cell might recruit to face zinc shortage for more than a limited time. Secondly, such an acute zinc loss rarely develops in human body as a result of a single short-term impulse. In fact, zinc deficiency, whether subchronic or chronic, has a slow, creeping character, and it is nowadays recognized that it takes time to reach this state, in particular when considering the complex interactions between various involved factors (Chai *et al.* 1999; Vallee & Falchuk 1993). Low-serum (1%) or serum-free medium reduced the intracellular zinc content in Hep-2 cells by 50% during 5 days cultivation, and after 10 days the intracellular zinc levels were at minimum (Figure 1). The observed morphological and growth changes in the exposed cells (cell elongation or shrinkage, development of vacuoles and decreased proliferation), which were first noticeable at the third day of cultivation, with subsequent acceleration over next seven days, confirm the findings of previous studies (Cario *et al.* 2000; Leure du Pree *et al.* 1982; Meerarani *et al.* 2000; Truong Tran *et al.* 2001). Of course, the results of this experiment have to be interpreted carefully as lowering serum deprives the cells of many important molecules, thereby slowing cell proliferation, and, often promoting untimely cell death (Kulkarni & McCulloch 1994). In attempt to minimize this interfering factor we exchanged cultivation media more often (twice a day instead of once two to three days) and kept initial cell seeding density somewhat higher than usual. Moreover, we followed growth and proliferation profile of treated cells which was generally slowed but not halted. At the onset of the first changes in cells' morphology and growth characteristics (the third day of our experiment), we treated a half of the exposed cell population to 15 μM zinc sulfate and the other half continued without any zinc supplementation. We noted that the cells treated with zinc sulfate were able to continue in proliferation and the morphological and growth changes regarded as harbingers of cell degeneration and death were found sporadically (somewhere between 5–10% of the cell population) in 10 days treatment model. Non-treated cells, on the other hand, deteriorated very rapidly and at the 10th day were practically dead. Still, these data are preliminary and we would need to specify them but it seems that zinc might play considerable role in maintaining Hep-2 cells populations viable despite the drastic reduction in supply of many other important molecules from serum. It is logical that the used model has limitations as in the longer time intervals the supplementation with zinc of Hep-2 cells would not be sufficient to cover the lack of other important molecules and the cells would most probably die due to the lack of basic nutrients and combination of other fac-

tors. The timing and dynamics of the observed process suggest its close association with tolerable reduction of intracellular zinc concentration. Hep-2 cells seem to momentary support this relatively drastic decrease (in close order to 75%) by limiting their metabolic and cell cycle activities as suggested by reports showing that zinc deficiency as well as excess may lead to cell cycle disturbances (Liang *et al.* 1999). On the other hand, it is interesting to note that even in the zinc-deprived cell population there were individual cells that appeared to be more resistant to such a stress and were able to survive and in a limited way proliferate for a longer time periods (data not shown). This ability has probably something to do with a selective adaptation potential of Hep-2 cells and we are planning to investigate this phenomenon more closely in the future.

Intracellular zinc depletion has also been shown to lead to the activation of several caspases (for example caspase-8 and -3), with subsequent entry of cells to apoptosis (Chimienti *et al.* 2001). Central to the execution of apoptotic cascade is the conversion of the inactive procaspase-3 into its active form, which appears early in the cytoplasm of zinc-deprived cells as demonstrated by recent studies (Chai *et al.* 1999; Nakatani *et al.* 2000; Takahashi *et al.* 1996). In our experiments, we tried to follow the dynamics of caspase-3 activation in Hep-2 cells exposed to low zinc environment and to correlate it with measured intracellular zinc content. We found that the amount of the active caspase-3 increases in a time-dependent manner, with a maximum reached at day 10 of cultivation (data not shown). Significantly higher number of caspase-3 positive cells as compared with control culture was first observed between days 5 and 6, which correlates with the approximately 50% reduction in zinc content (Figure 1). These results are in accordance with other reports (Chimienti *et al.* 2001), however, it is worth noting that at maximum only about one third of the treated cell population was tested positive for caspase-3 presence. Here we assume that despite the extensive depletion of intracellular labile zinc pools, which are presumed to be critical for induction or suppression of apoptosis (Chai *et al.* 1999), Hep-2 cells were able to maintain an inactivated procaspase-3, at least for certain time, possibly by recruiting zinc ions from the plasmatic membrane, cytoskeleton or nucleus. This hypothesis is supported by a range of observed morphological changes in the zinc-deprived cells, including cytoskeletal disturbances, cell membrane alterations and nuclear structure collapse (Figures 9, 10 and 11). In addition, it may be hypothesized that in our model the gradual labile zinc loss is more tolerable since it allows the exposed cells to employ stress-adaptation mechanisms, such as decreased rate of metabolism or slowed progression throughout cell cycle. That this process is not irreversible even after 10 days was demonstrated by supplying 15 μM zinc sulfate to the treated cells, which were quickly restored to their normal state within two days (data to be published).

Next, we have investigated the extent of zinc uptake and the effect of various zinc concentrations on morphology as well as physiological performance of Hep-2 cells. Laryngeal cells exposed to extracellular zinc during three days were found to accumulate it at differing rate, with the highest achieved intracellular zinc concentration being at 5.18 μg/mg protein reached at day 2 of supplementation with 100 μM zinc sulfate (Figures 2 and 3). This level seemed to be a maximum because it has not further increased in time (Figure 3). It has been experimentally verified that zinc accelerates cell growth by stimulating DNA synthesis in endothelial cells (Kaji *et al.* 1994); however, its effect on epithelial cells seems to be inconclusive as there are papers reporting conflicting findings (Cario *et al.* 2000). Our results indicate that the proliferation of Hep-2 cells was not significantly influenced by physiological zinc concentrations; the only exception was the lowest concentration (1.5 μM), which seemed to enhance the growth of laryngeal cells during the three-days period (Figure 8).

Zinc has been experimentally proven as cytoprotectant, stabilizing the microtubular cytoskeleton and cell membranes (O'Dell 2000). On the other hand, several studies aimed at the role of zinc in the nervous system concluded that zinc at higher concentrations might act as toxin, promoting neurodegeneration and perhaps development of Alzheimer disease through oxidative damage (Huang *et al.* 2000; Choi *et al.* 1988; Kim *et al.* 1999). In our model, the highest tested dose of zinc (750 μM) induced rapid changes in epithelial cells morphology and behavior. On the digitalized sequences it was possible to observe complete cessation of cell membranes ruffling as well as intracellular transport followed by overall cell collapse. This process was conceivably also originating from a severe alteration of cytoskeleton as the affected cells gradually shrank, leaving some of their anchor fibers adherent, and releasing cell membrane fragments, which might be in contact with these fibers (Figure 9). Thus in the light of our results it is pos-

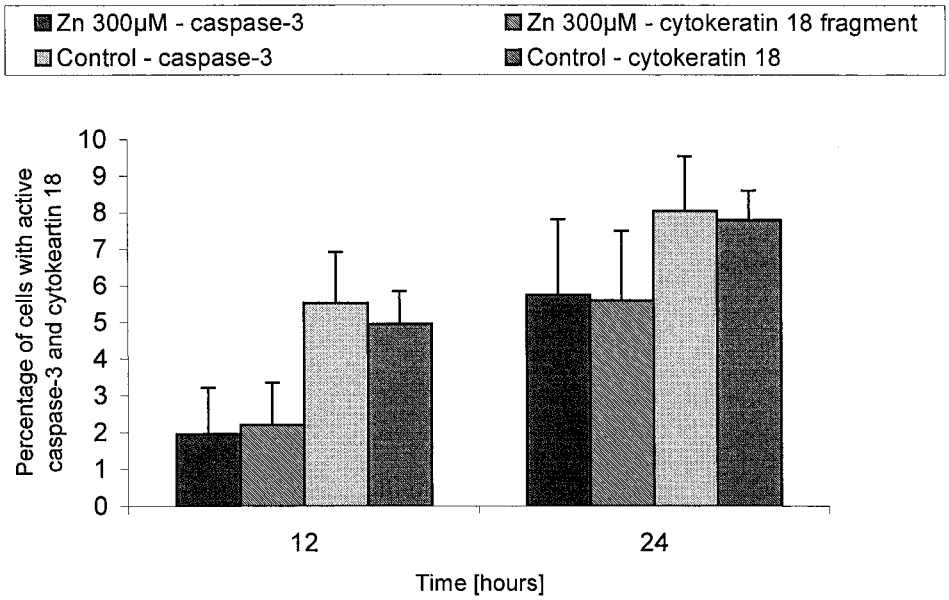

Figure 15. The influence of zinc sulfate at concentration of 300 μM on activation of caspase-3 and presence of cytokeratin fragment 18. Data represent the mean $\pm$ S.D. of three independent experiments.

sible to state that when the local zinc concentrations reach high supraphysiological levels its role might be reversed and instead of stabilizing it promotes an irreversible disruption and damage. Furthermore, our observed significant decline in cells metabolic activity in the first hour of treatment as measured by WST-1 assay as well as fluorescence staining of nuclei and caspase-3 assay confirmed that zinc at the above mentioned concentration induced necrosis in Hep-2 cells.

While the range of the observed effects of 300 μM zinc sulfate on Hep-2 cells resembled those described in the previous paragraph, with the exception of the timing of the entire process which was in this case shifted towards longer time periods (data not shown), the treatment of cells with 150 μM zinc sulfate resulted in observed apoptosis as confirmed by membrane blebbing, chromatin fragmentation and caspase-3 as well as CK 18 assays (Figures 11, 14 and 16). The fact that zinc was able to induce apoptosis in Hep-2 cells concurs with the similar results of other studies (Cario *et al.* 2000; Haase *et al.* 2001; Liang *et al.* 1999).

The ability of zinc ions at the concentration of 150 μM to activate caspase-3 seemingly contradicts observations of numerous published papers (Provinciali *et al.* 1995; Wolf & Eastman 1999; Wolf *et al.* 1999), which announced that zinc prevents maturation of this cysteine protase. Still, when analyzed, our results show that the presence of the active caspase-3 was significant only at one measured period (36 h), corresponding to a demonstrably damaged cell membrane, which could not therefore regulate the intracellular concentration of zinc ions, thereby leading to potentially high oscillations in intracellular zinc levels. It is thus possible that zinc ions by damaging the cell membrane structure triggered the apoptotic pathway, which could have advanced up to caspase-3 activation without preventing it due to momentary intracellular zinc deprivation. However, since after 48 h the number of caspase-3 positive cells was significantly lower than in control cultures, it may be argued that the observed caspase-3 activation was likely highly transient process occurring at one time period only. Further experiments are thus needed to provide more details pertaining to the impaired intracellular labile zinc regulatory mechanisms and corresponding biochemical changes in the exposed cells.

Conclusions

Epithelial Hep-2 cells maintain relatively steady intracellular zinc levels upon standard cultivation conditions. External zinc deprivation as well as supplementation causes statistically significant decrease or increase in these levels, respectively.

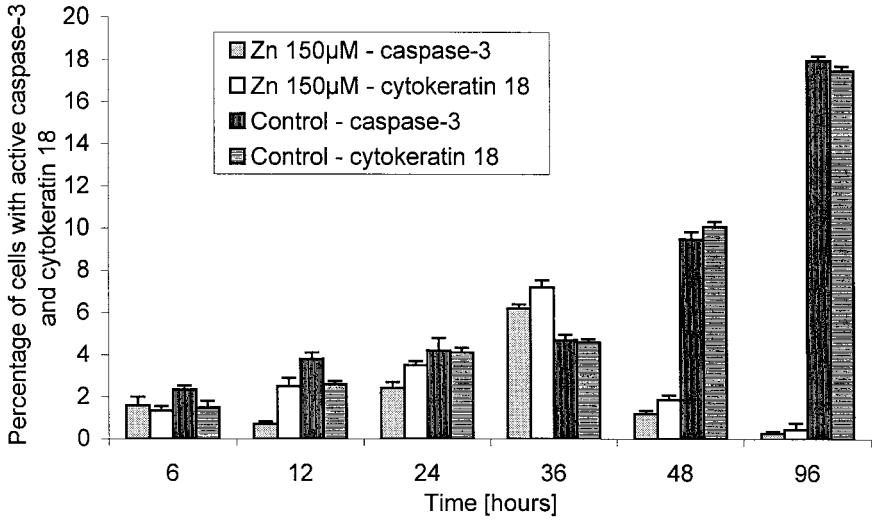

Figure 16. The influence of zinc sulfate at concentration of 150 μM on activation of caspase-3 and presence of cytokeratin fragment 18. Data represent the mean $\pm$ S.D. of three independent experiments.

Zinc shortage induced by low-serum environment appears to endanger laryngeal cells in two ways; by causing their premature death through apoptosis as well as by rendering them more susceptible to environmental stresses, with possible risk of malignant transformation. Nevertheless, individual cells that are more resistant to such a zinc shortage appear in the treated cell populations, and this resistance may be the selective advantage for them. The experimental results of this model, however, require a very careful interpretation, as it is very difficult to single out the effect of zinc alone on treated cell populations when low-serum environment is used. Furthermore, the above mentioned conclusions should be regarded as time and particular cell type-depending and more experiments are certainly needed to elucide this problematics.

Zinc supplementation has an ambiguous effect on laryngeal cells. Zinc concentrations of 1.5–100 μM do not interfere with the normal functioning of the cells, with the concentration of 1.5 μM slightly enhancing their proliferation rate. On the other hand, concentrations above 100 μM (150–750 μM) induce cell death which was identified to bear features of either apoptosis and necrosis, depending on the employed zinc concentration.

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