Change of Zinc Uptake under Growth Arrest and Apoptosis

ATSUSHI TAKEDA¹, HARUNA TAMANO¹ and YUKO IBUKI²

¹Department of Medical Biochemistry, School of Pharmaceutical Sciences and
²Laboratory of Radiation Biology, Graduate School of Nutritional and Environmental Sciences,
University of Shizuoka, Shizuoka 422-8526, Japan

Abstract. Zinc uptake is critical for cell proliferation. On the basis of the evidence that brain tumors are positively-imaged with ⁶⁵Zn, cellular zinc uptake was studied under growth arrest and apoptosis to understand the relationship between cellular viability and zinc uptake. When NIH3T3 cells were cultured in albumin-coated dishes under the presence of serum, the viability of the cells detached from the extracellular matrix, which was determined with fluoresceine diacetate, was almost the same as the control cells cultured in untreated dishes. Both the uptake of ¹⁴C-thymidine and ⁶⁵Zn by the cells was significantly suppressed by detachment from the extracellular matrix, suggesting that cellular zinc uptake is suppressed by growth arrest. When apoptosis was induced in the cells detached from the extracellular matrix under serum-free condition, ⁶⁵Zn uptake by the cells led to apoptosis which was significantly higher than that by the control cells. ⁶⁵Zn uptake by C6 glioma cells, which were irradiated with γ-ray, was also higher than that by control (unirradiated) C6 glioma cells. The present study demonstrates that zinc uptake is involved not only in the process of cell proliferation, but also in the process of apoptosis.

Zinc, an essential trace metal for animals and humans, has three functions in zinc metalloproteins, i.e., catalytic, coactive (or cocatalytic) and structural (1). Zinc is necessary for DNA replication and transcription, and protein synthesis. Numerous zinc enzymes and proteins are associated with the metabolism of proteins, nucleic acids, carbohydrates and lipids (2). This metal has critical roles in the regulation of proliferation and differentiation of cells. Dietary zinc deprivation powerfully retards the growth of humans and animals (3,4). It also effectively suppresses the proliferation of transplanted tumors in tumor-bearing animals (5-7). The response of neoplastic tumors to zinc deficiency indicates a potential for therapeutic opportunities.

On the other hand, zinc metabolism in tumor cells might be associated with their malignancy. Both normal and malignant cells secrete matrix metalloproteinases, which are important for invasion and metastases. The amount of the enzymes secreted by malignant cells is reported to exceed that of normal cells (8,9). In tumor cells, the functions of zinc are closely related to metastasis and proliferation. Zinc might also be involved in the regulation of the programmed cell death, apoptosis, which is observed in tumor cells treated with γ-irradiation and anti-tumor drugs (10). Thus, it is important to evaluate the relationship between cellular viability and zinc uptake, because there is the possibility that zinc uptake is an index of tumor viability (11).

The evidence that brain tumors are positively-imaged with ⁶⁵Zn suggests that ⁶⁹mZn, a short half-life gamma emitter (half-life, 13.76 h; energy, 439 keV), is useful for the diagnosis of brain tumors (12). Because the information obtained from radioactive zinc imaging is considered to be unique and important for the diagnosis and therapy (13), it is necessary to understand variations of zinc uptake by brain tumors. Such understanding is also important to clarify the functions of zinc in cells.

When NIH3T3 fibroblasts are cultured in albumin-coated dishes, the growth of the cells is arrested because of detachment from the extracellular matrix (14,15). The NIH3T3 fibroblasts detached are subjected to apoptosis under serum-free condition. NIH3T3 fibroblast is useful as an experimental tool to evaluate the relationship between cellular viability and zinc uptake, because the cellular viability can be modulated by a simple change of culture conditions (16). The present paper deals with zinc uptake by NIH3T3 cells under the conditions described above and by C6 glioma irradiated with γ-ray to understand the meaning of zinc uptake.

Materials and Methods

Chemicals. ⁶⁵ZnCl₂ [85.1 MBq (2.30 mCi)/mg] in 0.5 N HCl and [2-¹⁴C]thymidine [>1.85 GBq (50 mCi)/mM] in aqueous solution were obtained from PerkinElmer Life Sciences, Inc. (Boston, MA, USA).
Cell culture. NIH3T3 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) containing 10% heat-inactivated fetal bovine serum, penicillin G (100 U/mL) and streptomycin (100 µg/mL), in a humidified atmosphere of 5% CO2 and 95% air at 37°C. Astrocytoma C6, glioma cells, were maintained in minimum essential medium (Nissui Pharmaceutical Co., Ltd), containing 10% heat-inactivated fetal bovine serum, penicillin G (100 U/mL) and streptomycin (100 µg/mL), under the conditions described above.

Uptake of 14C-thymidine and 65Zn by NIH3T3 cells. Tissue culture dishes (35 mm) were treated with 1 mL of heat-denatured bovine serum albumin (2 mg/mL) at 37°C for 1 h. NIH3T3 cells (2 x 10^6 cells) in the media containing fetal bovine serum were seeded on untreated dishes and albumin-coated dishes, NIH3T3 cells (2 x 10^6 cells) in media without fetal bovine serum were also seeded on albumin-coated dishes. The NIH3T3 cells were incubated in a humidified atmosphere of 5% CO2 and 95% air at 37°C for 2-6 h. Ten µL of 14C-thymidine (1 µCi) or 65ZnCl2 (0.4 µCi) was added to the NIH3T3 cells in untreated dishes and albumin-coated dishes, and incubated for 1 h under the same conditions. The media were removed from the dishes and the NIH3T3 cells were washed with 1 mL of ice-cooled phosphate-buffered saline (PBS) twice. The radioactivity of 14C and 65Zn taken up by the cells was determined with a liquid scintillation counter (Aloka, LSC-3500) and γ-counter (Packard, MINAXI 5000), respectively. To subtract non-specific cell-associated radioactivity from the radioactivity taken up by the
cells, 10 µL of 14C-thymidine (1 µCi) or 65ZnCl₂ (0.4 µCi) was also added to the NIH3T3 cells in untreated dishes and albumin-coated dishes. The media were immediately removed and the radioactivity was measured according to the procedure described above.

Uptake of 65Zn by C6 glioma cells. C6 glioma cells were seeded at 2 x 10⁵ cells/35-mm tissue culture dishes. Five days after culture, the C6 glioma cells, which were subconfluent in the dishes, were irradiated with γ-ray (0.3 Gy/min) for 20 or 40 min. Twenty-four h after irradiation, 10 µL of 65ZnCl₂ (0.4 µCi) was added to the glioma cells, and incubated for 1 h under the conditions described above. The C6 glioma cells were washed with 1 mL of ice-cooled PBS twice, trypsinized and washed with 1 mL of the media twice. The radioactivity of 65Zn taken up by the cells was determined in the same manner.

Results

NIH3T3 cells were cultured for 6 h in albumin-coated dishes under the presence of serum. The viability of the cells detached from the extracellular matrix was determined by measuring the size of the cells and the intensity of fluoresceine diacetate taken up by the cells. Fluoresceine diacetate, a substrate of esterases, is entrapped in living cells. The viability was almost the same as the control cells cultured in untreated dishes and the rate of living cells was more than 90% (Figures 1a,1b,2a,2b). However, the uptake of 14C-thymidine by the cells detached from the extracellular matrix was significantly lower than that by the control cells.
The uptake of 65Zn was also significantly lower in the cells detached from the extracellular matrix than that in the control cells (Figure 3).

To induce apoptosis in NIH3T3 cells detached from the extracellular matrix, the NIH3T3 cells were cultured under serum-free condition. The viability of the cells cultured for 2 h was almost the same as the control cells and the rate of living cells was more than 90% (Figure 1c,2c). However, 65Zn uptake by the cells cultured for 2 h was significantly higher than that by the control cells (Figure 3). On the other hand, the viability of the cells cultured for 6 h was lowered unlike the culture under the presence of serum and the rate of living cells was approximately 45% (Figure 1d,2d). 65Zn uptake by the cells cultured for 6 h was also significantly higher than that by the control cells, while it was almost the same as the cells cultured for 2 h (Figure 3).

65Zn uptake by C6 glioma cells irradiated with γ-ray was also higher than that by unirradiated cells (Figure 4). The rate of living cells was more than 90% at both doses of 6 and 12 Gy (data not shown).

Discussion

Evaluation of the susceptibility of tumor cells to treatment is required after chemotherapy and radiation therapy (17). The growth of tumor cells may be arrested by such a treatment, and tumor cells may be led to necrosis and/or apoptosis (18). NIH3T3 cells are led from growth arrest to apoptosis by simple changes of the culture conditions (16). The present study took advantage of changing the culture conditions. When NIH3T3 cells were cultured in albumin-coated dishes under the presence of serum, the viability of the cells detached from the extracellular matrix was almost the same as the control cells cultured in untreated dishes. On the other hand, both the uptake of 14C-thymidine and 65Zn by the cells was significantly suppressed by detachment from the extracellular matrix, suggesting that cellular zinc uptake is suppressed by growth arrest.

When NIH3T3 cells detached from the extracellular matrix are cultured under serum-free condition, the cells are led to apoptosis. The viability of NIH3T3 cells detached was lowered time-dependently under serum-free condition.
indicating that the cells are subjected to apoptosis, as reported previously (16). $^{65}$Zn uptake by the cells led to apoptosis which was significantly higher than that by the control cells. In contrast, there was no appreciable difference in $^{65}$Zn uptake between the early stage (2-h culture under serum-free condition) of the process of apoptosis, at which the rate of living cells was almost the same as the control, and the proceeding stage (6-h culture under serum-free condition), at which the rate of living cells was approximately half of the control. Protein synthesis is involved in apoptosis, as well as cell proliferation. Apoptosis also requires a proteolytic system involving a family of cysteine-dependent aspartate-specific proteases, the caspases (19-21). The activation of these enzymes does not occur 2 h after the start of the culture under serum-free condition. Their activity reaches a maximum around 6-9 h after the start of the culture under serum-free condition (16,22). Thus, zinc uptake may be involved in the initiation and progress of apoptosis (10).

$^{65}$Zn uptake by C6 glioma cells, which were irradiated with γ-ray, was also higher than that by unirradiated C6 glioma cells. The irradiation is considered to be sufficient to suppress proliferation of C6 glioma cells (23) and the cells appeared to be led to apoptosis and/or necrosis. These results suggest that zinc uptake is involved not only in the process of cell proliferation, but also in the process of apoptosis.

Figure 4. Uptake of $^{65}$Zn by C6 glioma cells. C6 glioma cells were irradiated with γ-ray (0.3 Gy/min) for 20 or 40 min. Twenty-four hours after irradiation, $^{65}$ZnCl$_2$ was added to the glioma cells, and the uptake of $^{65}$Zn by the cells was determined 1 h after the incubation. Each bar and line represent the mean±SD (n=4). Asterisks indicate significant difference (*, p<0.001; t-test) from control (unirradiated) group.

Figure 4. Uptake of $^{65}$Zn by C6 glioma cells. C6 glioma cells were irradiated with γ-ray (0.3 Gy/min) for 20 or 40 min. Twenty-four hours after irradiation, $^{65}$ZnCl$_2$ was added to the glioma cells, and the uptake of $^{65}$Zn by the cells was determined 1 h after the incubation. Each bar and line represent the mean±SD (n=4). Asterisks indicate significant difference (*, p<0.001; t-test) from control (unirradiated) group.

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