Abnormal cell proliferation of *Euglena gracilis* by inorganic Cadmium and its prevention by trace mineral

Masumi WATANABE[1)], Takahiko HIGASA[2)], Kaoru NAKAMURA[3)],
Yukio KAWAMURA[4)], Isao KUDO[1)] and Tetsuya SUZUKI[1)]

[1)] *Hokkaido University, Graduate School of Fisheries Science, Hakodate,* 041-8611, *Japan;*

[2)] *Kyoto University, Research Institute for Food Science, Uji, Kyoto,* 611-0011, *Japan ;*

[3)] *Kyoto Universtiy, Institute of Chemical Research, Uji, Kyoto,* 611-0011, *Japan;*

[4)] *Akita Research Institute of Food and Brewing, Akita,* 010-1623, *Japan;*

Abstract

Cadmium (Cd) is a toxic heavy metal which causes cell impairment at higher concentration and acts as endocrine disruptor at extremely low concentration which may cause adverse effect against ecological systems involving humans. Studying toxic effect of heavy metals from the aspect of the cellular function by using a single cell eukaryotic protist *Euglena gracilis*, we revealed that an inorganic form Cd, $CdCl_2$ caused abnormal cell division at the ppb level that used to be regarded as safe level. In the present study, *Euglena gracilis* Z grown in the Koren-Hutner (KH) medium for 6 days at 28℃ under 2,800 lx light/dark cycle with every 12 hours interval was used as a biomarker organism. To the *Euglena* cells of 10^4 cell/ml in the Zn-deficient KH medium $CdCl_2$ as 200ppb to 20ppm was added, and cultured for 2 to 7 days under the same condition as above. The occurrence of teratogenic cell division was checked under Allen's video-enhanced contrast microscopy ARGUS-100. The effect of Zn addition on the prevention of $CdCl_2$-induced teratogenicity was examined with addition of $ZnSO_4$ from 1 to 200ppm by keeping $CdCl_2$ at 10ppm. Exposure of $CdCl_2$ on the *Euglena gracilis* Z cells caused abnormal cell proliferation under the Zn-deficient Koren-Hutner medium. More abnormal cell proliferation was observed at ppb level rather than at ppm level. As far as incubating the cells in the presence of $CdCl_2$ suppression of the outbreak of abnormal cell proliferation could not be accomplished even by the addition of Zn as 10 times as much molar as Cd.

Pollution by industrial waste was once a serious problem especially in Japan because it caused vari-

* 所在地：函館市港町3-1-1（〒041-8611）

ous health hazard such as 'Minamata disease' by organo-mercury, 'Itai-Itai disease' by cadmium (Cd), 'Kanemi Oil syndrome' by PCB and so forth[1]. Thank to strict regulation on dumping industrial wastes the pollution problem does not seem so serious as before. However, we are now facing an unexpected environmental pollution called 'endocrine disrupting chemical' (EDC) causing ecological threat and health hazard. The seriousness of the EDC is because the fact how EDC has affected the ecological system has remained unclear. The warning by Theo Colborn et al. in the 'Our Stolen Future' has gotten attention of public interest on the EDC[2]. So many synthetic chemicals have been revealed to affect endocrine functions through acting as hormone mimics, hormone blockers and indirect trigger to endocrinological disruption in humans and wildlife. The seriousness of EDC is due to their low dose effect, namely, their effect operates far below the safety level settled by the governments. For instance, the level of tributyl-tin (TBT) is regulated at ppm level, however, TBT affects the reproduction system of shell fish by causing imposex[3].

On the other hand, Cd exposure at high concentration shows renal toxicity and dysfunction of Ca metabolism in bone cell that causes severe osteoporosis known as 'Itai-Itai' disease in Zintsuu-river[1]. However, the threshold level of Cd as EDC causing element has not yet been fully understood. Because of industrial waste causing disorders such as 'Itai-Itai' disease and 'Minamata' disease, discarding Cd and Hg are totally banned in Japan. Even though it is highly possible that small amounts of heavy metals run out into the environment especially into marine environment. Studies on the EDC issue is intentionally focused on the endocrine aspect, and interest of researchers are mainly on the higher animals. However, concerning the fact that ecological system is constructed with great number of organisms from microorganisms to higher mammals in phylogenetic tree, we should not overlook the influence of EDC against small organism especially in marine ecosystem. Phytoplankton literally supports productivity of marine ecological system. To keep our earth healthy and expect stable food supply from the earth, we must pay attention to the marine small biota. Nakano et al. reported that $CdCl_2$ exposed to *Euglena* cells at 20ppm caused abnormal cell proliferation[4].

We have reported toxicity of a marine EDC, TBT, against a phytoplankton *Euglena gracilis* Z and physiologically functional material processed water as a remedy[5)-9)]. The present study was undertaken to examine the low dose effect of inorganic Cd on *Euglena gracils* Z and prevention of teratogenicity by zinc suuplementation to the medium.

Materials and Methods

Biomarker organism and Cd loading

Euglena gracilis Z was used as a biomarker organism to examine teratogenicity of $CdCl_2$ and its prevention by Zn. *E. gracilis* Z was grown under Koren-Hutner (KH) liquid medium with variable concentration of Zn at 28℃ of every 12 hrs light and dark intervals as reported in the previous

report[9]. Cells grown at early stationary phase were used for the experiment. To a 96 holes microtitration plastic dish, 150μl of KH liquid medium was added, followed by ca 10^3 cells were inoculated to the each well, namely, the hole number 1 to 12 in the rows A to H which contained normal KH liquid medium.

For assessing teratogenicity of Cd, *Euglena* cells were incubated with $CdCl_2$ of different concentrations ranging from 200ppb to 25ppm preliminarily added to the the KH liquid medium in the well.

Teratogenic cell proliferation was assessed by counting abnormal cell number in a video frame under the video-microscopy with Olympus IMT-2 inverted microscope equipped with ARGUS-100 (Hamamatsu Photonics, Hamamatsu, Japan). Counting of abnormal cell number was made at least with 10 frames over 12 holes.

Cell growth was monitored by reading turbidity of incubation medium in the small test tubes at 610nm everyday.

Assessing suppression of abnormal cell proliferation by Zinc

To assess whether zinc addition suppresses the Cd-induced abnormal cell proliferation or not, *Euglena* cells were grown under Koren-Hutner medium containing Cd (10ppm as $CdCl_2$) and zinc (as zinc sulfate) ranging from 1 ppm to 200ppm. Experiment was made under small scale using microtitration dish. Culture condition was the same as described above for $CdCl_2$ loading. To examine the effect of zinc in the oyster extracts, four kinds of zinc containing powder or paste (JCOE) as reported in the previous paper[10] were added to the medium in place of zinc sulfate. Other culture conditions were the same as above. After 5 days of incubation, cell morphology was examined with the inverted microscope (Olympus IMT-2) equipped with ARGUS-100.

Scanning Electron Microscopic Observation of the *Euglena* Cells

Fine cell sturucture of the *Euglena gracilis* Z grown under the various culture conditions was examined under the Hitachi Scanning Electron Microscope, S-2500. *Euglena* cells were fixed with 3% glutaraldehyde in potaassium phosphate-saline at 0～5℃ for 1 hr, followed by with 1% OsO_4 for 1 hr at room temperature. Dehydration was carried out with alcohol starting 30% to absolute alcohol with gradual increase of concentration, finally treated with tert-butyl alocohol. Prior to observation speciemen were coated with Pt. Observation was made at 1 kEv.

Results

Occurrence of abnormal cell proliferation

Figure 1 shows the occurrence of abnormal cell numbers under the different concentrations of $CdCl_2$. In the Figure 1 it is not necessarily obvious, however, outbreak of abnomal cells under different Cd concentration looks diphasic ; i.e., abnormal cell proliferation frequency is recognized around at 200ppb and 3.2ppm in Zn-sufficient KH medium, while outbreak peaks are seen around 800ppb and

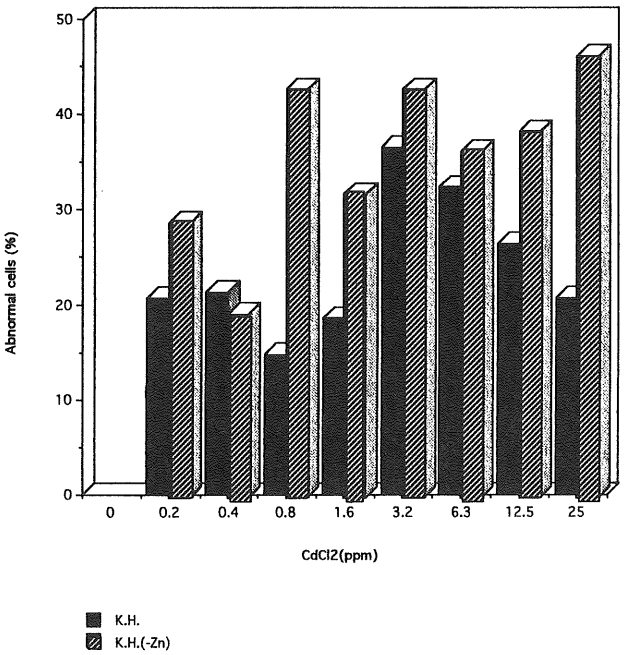

Fig. 1 Outbreak of teratogenic *Euglena gracilis* Z proliferation under different concentrations of $CdCl_2$ in Zn-sufficient or Zn-deficient Koren-Hutner media in whichi *E. gracilis* Z cells were grown under KH medium with increasing $CdCl_2$ concentrations.
E. gracilis Z cells were grown under Zn-deficient KH medium with increasing $CdCl_2$ concentrations from 0 to 25 ppm. Data are represetented as means of three measurements.

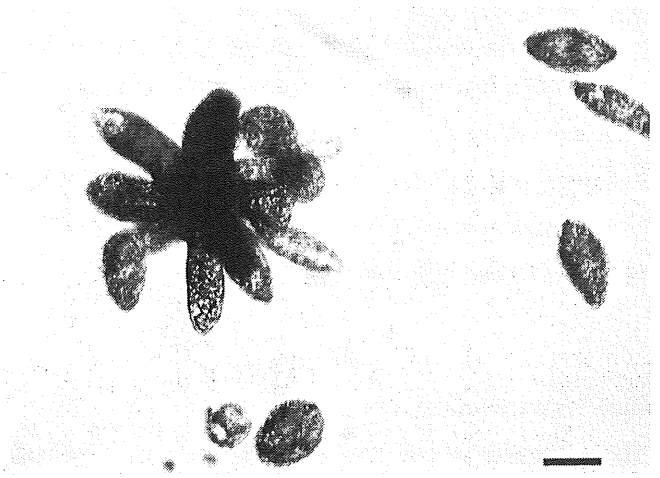

Fig. 2 Teratogenic *Euglena gracilis* Z cells grown under KH medium in the presence of 25 ppm $CdCl_2$ for 6 days bar : 50 μm

25ppm under Zn-deficient KH medium. More interestingly the cell morphology showed distinctively different, i.e., at ppm level imcompletely divided cells made up of two combined cells were frequently observed under the microscope. On the other hand, at ppb level starfish-like abnormal cells were more frequently observed (Figure 2). Nakano et al[4]. reported starfish-shape teratogenic cell at 20ppm $CdCl_2$. The difference between Nakano et al. and our data would be due to the different incubation media and incubation conditions. Nakano referred 1 ppm Zn level as zinc sufficient condition in their medium, however, as much as 25ppm Zn is contained in the KH medium. With this respect 1 ppm Zn level is definitely zinc-deficient, and we regarded Zn at 1ppm as Zn-deficient.

Suppressive effect of abnormal cell proliferation by zinc

Comparing the outbreak of abnormal cell proliferation by $CdCl_2$ under Zn-deficient (1 ppm) and Zn-sufficient (25ppm) conditions, it is certain that abnormal cell proliferation is less under Zn-sufficient condition than deficient one (Figure 1). However, increasing Zn concentration up to 200ppm did not completely suppress the outbreak of abnormal cell proliferation. Although detailed data are not presented in this paper, Zn suuplementation as much as 200ppm, or ca. 10 times molar concentration of Cd, did not completely suppress the outbreak of abnormal cell proliferation. Abnormally divided cells were still observed under the light microscope (Figure 3). In the presence of 10ppm $CdCl_2$, cell motil-

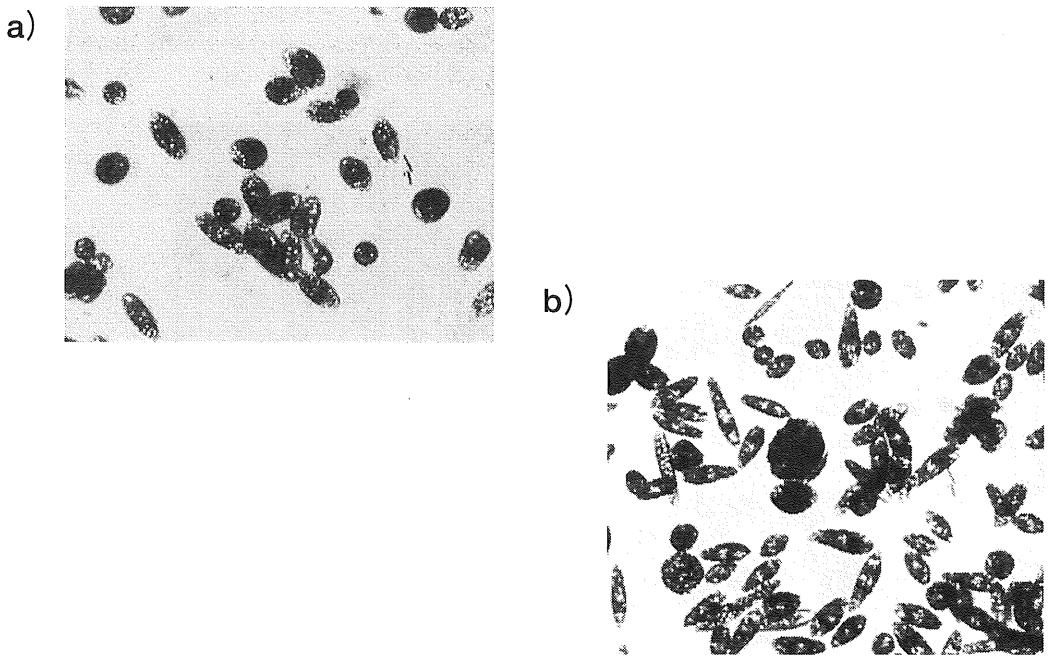

Fig. 3 *Euglena gracilis* Z grown under $CdCl_2$ with Zn supplementation
a) 25 ppm Zn + 10 ppm Cd ; b) 200 ppm Zn + 10 ppm Cd

ity was severely low, and the cholorophyllous *Euglena gracilis* Z cell grown under light/dark cycle became colorless meaning inhibition of chlorophyll formation. Supplement of Zn as much as 200ppm did not work to recover chlorophyll formation (data not shown), but cell motility was slightly recovered with increasing Zn concentration.

Discussion

What we tried in the present study was to examine how low level of Cd exposure would affect cell division and whether the Cd-induced abnormal cell proliferation of *Euglena gracilis* Z would be suppressed by a trace nutrient, Zn or not. In this study we found that the threshold level of outbreak of abnormal cell proliferation by Cd took place by exposure at as low as 200ppb under Zn-deficient condition, which used to be regarded as NOEL. Observation under the electron microscope showed that the cell surface structure of *Euglena* grown under Zn-deficient condition (1 ppm) was quite abnormal, i.e., rough and irregular structure (Figure 4). Under the culture condition in the presence of high $CdCl_2$ concentration (25ppm), morphology of the abnormal cells after 6 days incubation were mostly bottle gourd shape or v-shape indicating once or twice cell division before termination of the cell multiplication. It means that cell division terminated after once or twice cell division. On the other hand, Cd exposure at ppb level gave rise many starfish-like shape cells combining as many as 6 cells, which indicates cell division took place more than twice. Beside the starfish-like cell, round, fat and large-size cells were observed. The fact that starfish-shape teratogenic cells were more frequently

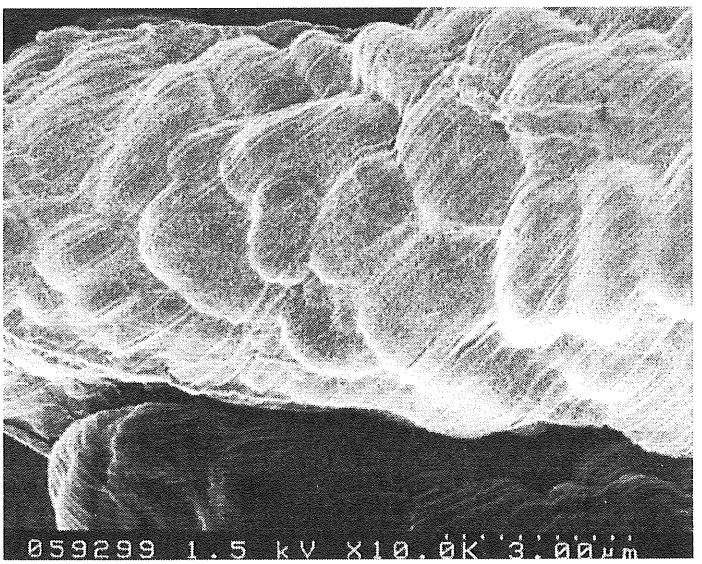

Fig. 4 Cell surface structure of *E. gracilis* Z grown under Zn-deficient Koren-Hutner Medium observed with Scanning Electron Microscope

observed in our experiment means cell cycle was not suppressed under ppb level.

Although it is just a hypothesis yet, obtained results mentioned above may mean necrosis took place under high concentration of Cd exposure. With extremely high concentration of Cd exposure over 20ppm, cell may suffer from necrosis, but Cd exposure at moderate level as 1 to 20ppm ***Euglena*** cell may recognize adverse environment around them, so that apotopsis-like reaction would operate. However, under extremely low concentration Cd exposure at ppb level cells do not recognize Cd as teratogen. Therefore, protecive mechanism did not operate to stop the abnormal cell proliferation. It is well recognized that ***Euglena*** cell is a eukaryote with highly developed subcellular organella equivalent to mammals. More interestingly, ***Euglena*** cell is extremely unique organism having both plant and animal characters. Consequently, what happens in the ***Euglena*** cells may happen in the mammals including human beings. Teratogenic cells brought about by abnormal cell proliferation are unable to keep their cell functions normal. Dysfunction of the cell may lead to extinction of the species. The fact that extra amount of Zn addition as $ZnSO_4$ to the medium could not completely suppress the outbreak of abnormal cell proliferation even at 10 times as much molar of Cd indicates the $CdCl_2$ acts on cell division so strongly. Considering the mechanism of Cd toxicity involves reactive oxygen and free radical species[11),12)], it may be extremely difficult to prevent the abnomal cell proliferation as far as Cd remains in the medium[13)].

In our previous paper, we reported that addition of oyster extract promoted restoration of TBTCl-intoxicated ***Euglena*** cell morphology and motility[10)]. However, we could not obtain preventive effect of oyster extracts on the outbreak of abnormal cell proliferation by $CdCl_2$. Because Zn in oyster is extremely high, we expected its preventive effect on Cd-induced teratogenicity. The reason why we could not obtain the positive data as expected remains unknown. It may be due to the purity of the oyster extract. We are planning to reexamine with purified oyster extract.

References

1) Miller, S.A., ; Amdur, M.O. ; Menzer, R.E. (1991) Environmental Toxicology, In "Casarett and Doulls' Toxicology, 4th Edition", (eds. Amdur, M.O., Doull, J., Klassen, C.D.), McGraw-Hill, Inc., New York, U.S.A.

2) Colborn, T., Dumanoski, D. and Meyers J.P. (1996) Our Stolen Future. Dutton, Penguin Books, U.S.A.

3) Horiguchi T ; Hyeon-Seo C ; Shiraishi H ; Shibata Y ; Soma M ; Morita M ; Shimizu M (1998) Sci Total Environ, 214 : 65

4) Nakano, Y., Abe, K. and Toda, S. (1980) Agric. Biol. Chem. 44 : 2305

5) Suzuki, T., Takahashi, N., Fukunaga, K., Okamura, M., Tanaka, T., Takama, K. (1993) Environ. Toxicol. Water Qual. 8 : 207

6) Suzuki, T., Doi, H., Shimono, I., Takama, K. (1995) Trace Nutr. Res. 12 : 171

7) Suzuki, T., Ikeuchi, R., Takama, K. (1996) Trace Nutr. Res. 13 : 105

8) Ohta, M., Nakamura, K., Takama, K. and Suzuki, T. (1996) Trace Nutr. Res. 14 : 95

9) Ohta, M., Nakamura, K., Kurihara, Y., Takama, K. and Suzuki, T. (1998) Trace Nutr. Res. 15 : 133

10) Suzuki, T., Ohta, M., Nakamura, K., Takama, K. (1997) Trace Nutr. Res. 14 : 103

11) Brennan, R.J., Schiestl, R.H. (1996) Mutat. Res. 356 : 171

12) Bagchi, D., Vuchetich, P.J., Bagchi, M., Hassoun, E.A., Tan, M.X., Thcng, L., Stohs, S.J. (1997) Free Radic. Biol. Med. 22 : 471

13) Shaikh, Z.A., Zaman, K., Tang, W., Vu, T. (1999) Toxicol. Lett. 104 : 137