CO6-1 Neutron Activation Analysis for Trace Elements in Scalp Hair of Patients with ALS

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INTRODUCTION: A high incidence of amyotrophic lateral sclerosis (ALS) and parkinsonism-dementia complex (PDC) has been found in the Koza/Kozagawa/Kushimoto (K) area and Hohara area of the Muro district in the Kii Peninsula of Japan. Environmental research revealed that drinking water in the K area showed low contents of Ca and Mg and the serum of residents and patients with ALS in the K area also showed low contents of Ca [1]. It is reported that Ca deficiency induces distributional changes of other metals in bones and soft tissues by secondary hyperparathyroidism. We speculated that a chronic Ca deficiency might induce an increase of absorption of toxic metals, and consequently increase metal-induced oxidative stress on neurons [2] and play a role in the development of ALS and PDC in the Muro district. To examine this hypothesis, we investigated the metal contents in the scalp hair of patients with ALS or PDC and residents in the Muro district.

EXPERIMENTS: Approximately 200 mg of hair was obtained from respective patients with ALS or PDC in the Muro district, residents in the Muro district and controls. The hair samples were washed with 50 ml acetone, 50 ml d.d.w. (3 times) and 50 ml acetone in sequence. The samples were then dried in air. Approximately 30 mg of the sample was weighed and double-wrapped in polyethylene films and subjected to NAA. The samples in polyethylene capsules were irradiated in Pn-1 of KUR (1000 kW) for 2 minutes as short irradiations and for 120 minutes as long irradiations. As comparative standards, orchard leaves (NBS) as well as elemental standards for Ca, Mg, Cu, Al, Mn, V, Br, Cl, S, Zn, and Hg were used. The γ-ray spectroscopic measurements with a Ge detector were performed repeatedly. The measurement time was 200 seconds for the short-lived nuclides after a cooling time of 110-320 seconds, and for the long-lived nuclides, it was 5000-6000 seconds after cooling for approximate one month.

RESULTS: 68 residents in the Muro district, 6 patients with ALS and 5 patients with PDC from the Muro district, and 22 residents from control area participated in this research. The contents of Ca, Mg, Al, Cu, Mn, Cl, Hg, V, S and Zn were determined in the hair samples. The Ca content was significantly influenced by hair treatments. The Mg content in hair samples from patients with ALS or PDC in the Muro district was significantly higher than those of controls (Fig.1).

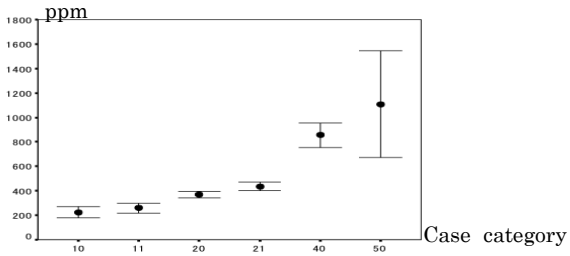

Fig. 1. The Mg content in hair samples was shown (ppm, Mean +- SE). 10: controls without hair treatments, 11: controls with hair treatments, 20: residents in the K area without hair treatments, 21: residents in the K area with hair treatments, 40: patients with PDC without hair treatments, 50: patients with ALS without hair treatments.

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REFERENCES:

[1] Kihira T, *et al.* NEUROLOGICAL MEDICINE 2010; 73: 507-51

[2] Rothstein JD. Ann Neurol 2009; 65 Suppl 1: S3-9

採択課題番号 23007　　紀伊筋萎縮性側索硬化症における金属イオンと酸化的ストレス障害　　共同通常

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CO6-2 Structure Specificity of Radio-Induced Biomolecule Damage and Its Effect Radio-Biological Consequence

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INTRODUCTION: Ionizing radiation causes various deleterious effect for living organisms. The effect is derived from damaging of biological molecules with ionizing radiation. Among numerous biological molecules, deoxyribonucleic acids (DNA) seem to be the most important ones as the only one genetic material. An ionizing radiation passes through (or along) the target DNA molecule as a beam. As passing, it leaves multiple damage close to the track. Such damage accumulation is termed as clustered DNA damage (CDD). CDD is considered as a damage aggregation containing two or more lesions in the two or three helices. The complex structure of CDD might lead to be its less repairability and high inhibition for DNA replication. CDD seems to be a major factor responsible to severity of radiobiological consequence [1]. In this study, we estimated the yields of CDD in the irradiated cultured cells to clarify the function of CDD in radiobiological effect.

EXPERIMENTS: Chinese hamster ovary (CHO) AA8 cells growing exponentially were irradiated by gamma-ray (0.2 keV/μm), and carbon (13 keV/μm), silicon (55 keV/μm), argon (90 keV/μm) and iron (200 keV/μm) particle beams, respectively (parenthetic numbers indicate respective LETs). The gamma-irradiation was at ^{60}Co-gamma-source at KURRI, and those heavy ion beams were obtained from HIMAC at NIRS. The irradiated cells embedded in agarose plugs were analyzed on agarose gel electrophoresis. For estimation of the total CDD, we summed DNA-double strand breaks (DSB) and oxidative base lesion clusters (BLC). The detail of analysis procedure was based on our previous report [2].

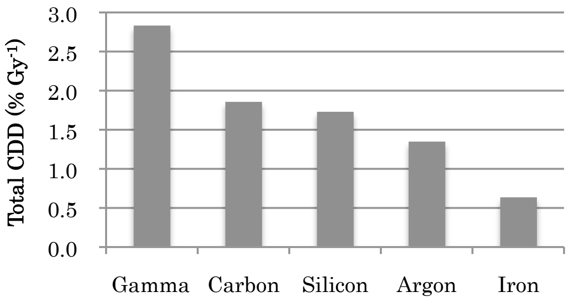

Fig. 1. The yields of clustered DNA damage (CDD) in the irradated cells.

RESULTS: The yields of total cellular CDD decreased as LET of the radiations grew (Fig. 1). On agarose gel electrophoresis, total damaged fractions of the chromosomal DNA in irradiated cells were 2.8%, 1.9%, 1.7%, 1.4% and 0.65% for gamma-ray, carbon, silicon, argon and ion particle beams, respectively. Similarly, the yields of total isolated DNA damage shows parallel pattern for the LETs (Fig. 2). Isolated damage were analyzed by ARP [2]. The total isolated DNA damage in the irradiated cells were 0.315, 0.206, 0.141 and 0.122 sites 10 bp^{-1} Gy^{-1} for gamma-ray, carbon, silicon and argon particle beams, respectively. The data of isolated damage for iron particle beam is now under analysis. On the other hand, the survival fractions of the irradiated cells reduced for LET increasing. The D_{10} for gamma-ray, carbon, silicon, argon and ion particle beams, were 4.8, 3.7, 2.6, 2.4 and 2.2 Gy, respectively, which were from colony forming assay. Those results indicate that the yields of radiation DNA damage even the clustered DNA damage are not directly bound up with the severity of radiobiological effect. We previously demonstrated the similar results with *in vitro* analyses for plasmid DNA and phage DNA irradiated with various ionizing radiations including heavy ion beams [2]. Many other studies also indicated similar correlation between yields of DNA damage and LET of ionizing radiation, too. Therefore, we need to consider another factor for the nature of clustered DNA damage other than the quantity. These are thought to be the structure, repair efficiency and others.

Conclusively, the yields of radiation CDD showed inversely proportional to their LETs in irradiated cells. It suggests that yields of CDD are not simply responsible to severity of radiobiological consequence.

REFERENCES:
[1] H. Terato and H. Ide, Biol. Sci. Space, **18** (2004) 206-215.
[2] H. Terato *et al.*, J. Radiat. Res., **49** (2008) 133-146.

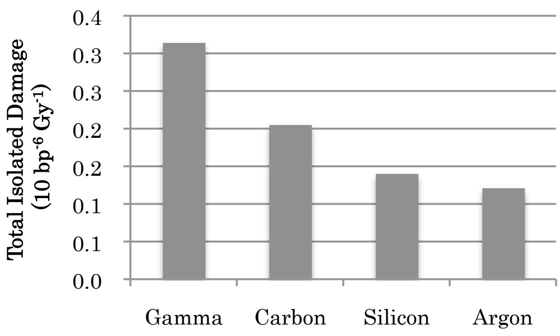

Fig. 2. The yields of isolated DNA damage in the irradated cells.

採択課題番号 23011　　放射線によって生じる生体構成分子の損傷構造の特異性と ” 析
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CO6-3 Determination of Trace Elements in Organs of Zn-deficient Mice

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INTRODUCTION: Zinc is one of the most important essential trace elements and it interacts with many other trace elements in living organisms. Our previous works have shown that the Co content increased significantly in all the organs and tissues of Zn-deficient mice, which had been fed with Zn-deficient diet for 1 week, 3 weeks, or further period from 3 or 8-week old, compared with those of mice fed with control diet, and that this fact suggested the partial substitution of Co with Zn in their metal proteins or other metal-bound compounds [1-4] . In the present work, concentrations of trace elements in organs of mice were determined in order to compare the effect of the zinc dosage of several kinds of chemical form on zinc deficiency.

EXPERIMENTS:

Animals and samples Male mice of the ICR/jcl strain, 8-week old, were divided into five groups. One group was fed with Zn-deficient diet and ultra pure water (Zn-def. mice) for two weeks. The other one group was fed with control diet (the diet was made with raw materials for Zn-deficient diet and Zn as zinc carbonate, basic) and the same water for two weeks. Remaining three groups were fed with Zn-deficient diet for a week. Then, they were fed with control diet described above or the other two kinds of specially prepared control diet in which Zn was added as zinc gluconate or zinc sulfate.

After the treatment, their livers, pancreata, and testes were removed under diethyl ether anesthesia. The removed organs were homogenized with HEPES buffer. In this process, the pancreata and testes of every four mice of each group were together homogenized because each pancreas or testis is too small for analysis. Cytosolic fractions were separated by differential centrifugation at 105,000×g for 65 min. They were weighed, freeze-dried, weighed again and grounded. Each sample (10 - 50 mg) was doubly wrapped in a polyethylene film and subjected to INAA(Instrumental Neutron Activation Analysis).

INAA The samples in polyethylene capsules were irradiated in Pn-1 for 6 minute and for 4 hours, for short and long irradiation, respectively. The γ-ray spectroscopic measurements with an HPGe detector were performed repeatedly for the short-irradiated samples: the first measurements for 120 – 300 seconds after decay time of 5 - 10 minutes and the second one for 250 - 900 seconds after 60 - 150 minutes. The long-irradiated samples were measured for 6 - 24 hours after an adequate cooling time (15 - 80 days).

RESULTS: Content of ten elements, Na, Mg, Cl, Mn, Fe, Co, Cu, Zn, Se and Rb, were determined. Zinc content in cytosolic fraction of livers and testes of Zn-defcient mice were not lower than those of control mice or the mice which were fed with Zn-added diet after feeding with Zn-deficient diet. The content in cytosolic fraction of pancreatic cells of Zn-deficient mice were distinctly lower than those of the other mice. On the other hand, the data of Co content indicates that one-week zinc dosage was insufficient for recovery from zinc deficiency as shown in Fig. 1.

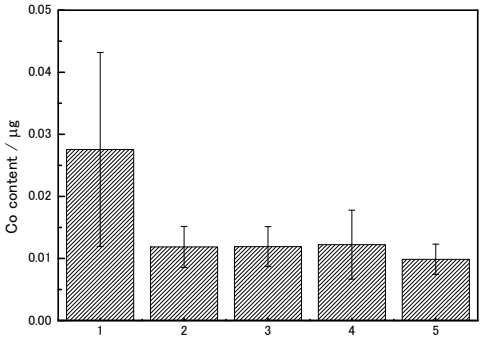

Fig. 1 Cobalt content in cytosolic fraction of livers of various kinds of mice;
1: Zn−def. diet 2: Zn−def.diet + control diet 3: Zn−def. diet + ZnSO4 included diet
4: Zn−def. diet + zinc gluconate 5: control diet

REFERENCES:

[1] M. Yanaga, M. Iwama, K. Takiguchi, M. Noguchi and T. Omori, *J. Radioanal. Nucl. Chem.*, **231** (1998) 187-189.

[2] M. Yanaga, M. Iwama, K. Shinotsuka, K. Takiguchi, M. Noguchi and T. Omori, *J. Radioanal. Nucl. Chem.*, **243** (2000) 661-667.

[3] M. Yanaga, H. Wakasa, T. Yoshida, M. Iwama, K. Shinotsuka, M. Noguchi and T. Omori, *J. Radioanal. Nucl. Chem.*, **245** (2000) 255-259.

[4] T. Ogi, Y. Kawamoto, H. Maetsu, M. Koike, T. Ohyama, S. Enomoto, M. Noguchi, H. Suganuma and M. Yanaga, *Proc. Intern. Symp. on Bio-Trace Elements 2002* (2000) 83.

採択課題番号 23023 亜鉛欠乏マウス臓器中の微量元素の分析 共同通常
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CO6-4

New Reagents for Disulfide-Coupled Protein Folding

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INTRODUCTION: The disulfide bond formations are important not only for the folding of secreted proteins and peptides, but also for the stabilities of their tertiary structures for the expression of biological activities. Such reactions are thermodynamically and kinetically related to the redox reagents and their redox potential [1], for instance, glutathione, cystein, protein disulfide isomerase (PDI), and their relatives (Dsb proteins). These reagents catalyze both the formation and isomerization of disulfide bonds *in vivo* or *in vitro*. Recently, we demonstrated that positively charged redox reagents are preferred for accelerating the disulfide-exchange reactions, as evidenced by the fact that the folding recoveries by using such reagents are greater than that by using a typical redox system [2]. Although the formation of disulfide bonds and the tertiary structure of a target protein is affected by a designed redox reagent, the nature of the redox environment its relationship with protein folding remains a matter of debate. The objective of this study was to develop the effective disulfide-coupled folding method by using new additives as a redox reagent.

EXPERIMENTS: *Peptide Synthesis*- Two kind of peptides (peptide A and peptide B) were synthesized by the Fmoc solid-phase method by using a PSSM-8 peptide synthesizer (Simadzu, Kyoto). The resulting peptides, containing two cystein residues, were air-oxidized to form an intramolecular disulfide bond and the product was purified by RP-HPLC (Hitachi High-Technologies, Tokyo). The purified peptides were dissolved in 0.1 M Tris/HCl buffer (pH 8.0).

Preparation of reduced/denatured proteins- The reduced/denatured proteins were prepared according to previous method [2], that is, proteins, hen egg white lysozyme (HEWL), bovine pancreatic trypsin inhibitor (BPTI), and prouroguanylin, were dissolved in 0.1 M Tris/HCl (pH 8.3) containing 20 mM dithiothreitol and 8 M urea, and the solutions were kept at room temperature for 3 hours. The reaction mixtures were then dialyzed against 10 mM HCl and lyophilized.

Kinetic analyses- The refolding reaction of HEWL was performed in 0.1 M Tris-HCl (pH 8.0) buffer containing 1.0 mM GSH and 0.2 mM GSSG in the presence or absence of 1 mM the synthesized peptide (peptide A or B). Correctly folded HEWL has bacteriolysis activity, a *Micrococcus luteus* suspension (0.5 mg/mL) in 50 mM phosphate buffer (pH 6.5) was prepared to measure the activity. The bacteriolysis reaction was started by mixing 10 μL of the refolded HEWL solution and 1 mL of a *Micrococcus luteus* suspension, and was quenched at several time-points by adding a quenching solution containing 0.5 M mono-iodoacetate, 1 M KOH, 1 M Tris/HCl buffer (pH 7.0). The light scattering intensities of the reaction mixtures were measured at 600 nm.

Oxidative folding analyses- The denatured/reduced proteins were dissolved in 0.1 M Tris/HCl (pH 8.0) and allowed to undergo folding in the presence of 2 mM reductant and 1 mM oxidant at room temperature for 48 hours, as described previously [2]. All solutions used in the refolding experiments were flushed with N_2 gas, and the reactions were carried out under an atmosphere of N_2 in a sealed vial. The reaction mixtures were sampled at several time-points, quenched with an equivalent volume of 1 M HCl [2], and separated by RP-HPLC. The HPLC fractions were analyzed by MALDI-TOF/MS after lyophilization.

RESULTS: To estimate the effects of the synthesized peptides on protein folding, we employed HEWL, BPTI, and prouroguanylin as model proteins. Refolding yields of HEWL by using peptide A or B were greater than that by using a typical glutathione redox system, suggesting that folding velocity increased in the presence of the synthesized peptides.

HPLC analyses of disulfide-coupled folding of BPTI showed that the reduced/denatured and the scrambled-disulfide species (folding intermediates) of BPTI rapidly converted to native form with correct disulfide bonds as compared with glutathione redox system. It is considered that the synthesized peptides A and B accelerated the formation and the isomerization of intra-molecular disulfide bonds.

In general, an appropriate redox condition is necessary for correct folding of disulfide-containing proteins that have multiple disulfide bridges, because these proteins fold into the native conformations *via* the formation of complex disulfide intermediates. It is therefore a complicated problem to choose the suitable folding condition with redox environment. In our study, we demonstrated that the new redox molecules, peptide A and B, are preferred for accelerating the folding reaction and that the new method using them is effective in forming the native conformation of proteins. To investigate the further folding mechanism by using the new reagents, analysis using folding intermediates of prouroguanylin is in progress.

REFERENCES:

[1] J.L. Arolas, *et al., Trends Biochem Sci.,* **31** (2006) 292-301.
[2] M. Okumura *et al., FEBS J.,* **278** (2011) 1137-1114.

採択課題番号 23049　　新規リフォールディング試薬の開発　　共同通常

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CO6-5 Combination Therapy of BNCT and PDT Sensitized by A Boron-Porphyrin Derivative in a Glial Cancer Cell Line

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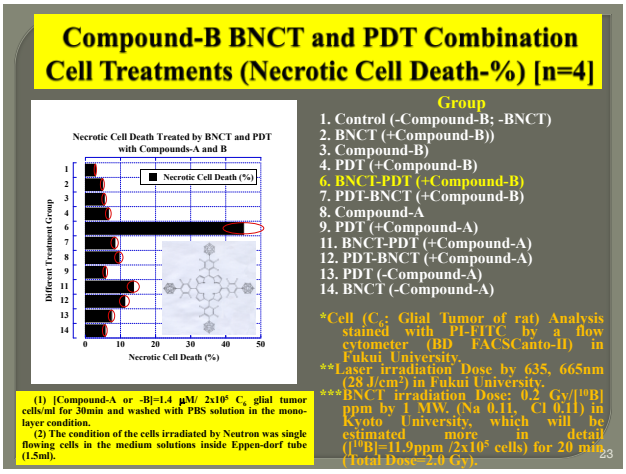
INTRODUCTION: Recently, the cancer treatment by BNCT has been taken the clinical trial widely in Kansai region and the near fields at the special institution, Research Reactor Institute of Kyoto University. Furthermore, the compact generator of neutron beam will be progressed in future, especially against the malignant tumor cases. It should stop it as the malignant tumor will repeat the re-growth of it combined with other treatment for example, photodynamic therapy (PDT) using the photosensitizer and a compact LD laser [1-2]. We had been developed the pulsed two wavelength of 635 and 665 using 5-aminolevulinic acid (5-ALA) [3-4]. In this machine time (14th Dec. 2011 and 17th Jan. 2012), we experimented combination treatments of BNCT and PDT of a glial cancer cell line (C6) using a boronated porphyrin derivative as the both sensitizer which was presented from Russia Cancer Center and Institute of Organoelement Compounds.

EXPERIMENTS: The irradiated cell samples were prepared with the 1.4μM sensitizer (compound-B) solutions for 30 min incubation in a CO_2 incubator before the both irradiations (2.1Gy for 20 min of BNCT and 150J/cm^2 for 17min of PDT, the duration time was 36 hr between both irradiations). The dose of neutron beam was finally calculated from the average value of the 7 detectors of gold leaf and thermo-luminescence dosimeter on the 24 Eppendorf sample tubes. The behind and sides of sample were sealed with 5 enriched lithium-6 fluoride plats (2mm thickness). The percentage of the necrotic cells stained with an apoptosis kit (annexinV-FITC and propidium iodide (PI), MBL Co. Ltd., Nagoya) were analyzed by a flowcytometer (BD FACS-Canto-II type, BD Sciences, Co. Ltd., Tokyo) after the 1 day incubation.

RESULTS and DISCUSSION: It was resulted that the combination therapy of a tumor cultivated cell line (C6) cells were effectively damaged to induce the necrotic cell death because the damaged cells were stained at higher percentages by propidium iodide as following graph in the lower slide.



Especially, it was large effects only when the BNCT treatment had done before the PDT one using the compound-B. The anti-cancer effect exchanged sequence was no effect. It was estimated that the damaged cell analysis was done after the incubation for over-night of the treated cells which were recovering from the light (small) damages.

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REFERENCES:

[1] N. Miyoshi, A. B. Bibin, et al., "i*n hoe of going over the present Clinical PD and PDT"*, Eds. by N. Miyoshi and R. H. Pottier, Sankeisha, Nagoya, ISBN978 -4-88361-883-5 (2011) pp.33-39.

[2] N. Miyoshi, K. Kume, et al., *International Proceedings,* Ed. by G. Kostron, Medimond, Italy (2011) pp.121-125.

[3] N. Miyoshi, K. Kume, et al., "*Aminolevulinic acid*" Eds. by I. Okura and T. Tanaka, ISBN978-4-7954-0229-4, Tokyo Inst. Tech. Press, Tokyo (2011) pp. 23-29.

[4] N. Miyoshi, T. Ogasawara, et al., "*Recent Progress of Clinical and Basic Research of ALA*" Eds. by N. Miyoshi and S. Kaneko, Kashiwaba Neurosurgery Hospital, Sapporo (2005) pp. 11-16.

採択課題番号23073　腫瘍特異性を増させた捕捉剤投与による BNCT と光線力学治療の併用療法による前臨床共同研究　　共同通常

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