Original Communication

The Oral Intake of Organic Germanium, Ge-132, Elevates α-Tocopherol Levels in the Plasma and Modulates Hepatic Gene Expression Profiles to Promote Immune Activation in Mice

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Received: December 19, 2014; Accepted: March 5, 2015

Abstract: The common water-soluble organic germanium compound poly-*trans*-[(2-carboxyethyl) germasesquioxane] (Ge-132) exhibits activities related to immune responses and antioxidant induction. In this study, we evaluated the antioxidative effect of dietary Ge-132 in the plasma of mice. Male ICR mice (seven mice per group) received an AIN-76 diet with 0.05 % Ge-132; three groups received the Ge-132-containing diet for 0, 1 or 4 days. The plasma alpha-tocopherol (α-tocopherol) concentration increased from 6.85 to 9.60 μg/ml after 4 days of Ge-132 intake ($p < 0.05$). We evaluated the changes in hepatic gene expression related to antioxidative activity as well as in the entire expression profile after one day of Ge-132 intake, using DNA microarray technology. We identified 1,220 genes with altered expression levels greater than 1.5-fold (increased or decreased) as a result of Ge-132 intake, and α-tocopherol transfer protein (Ttpa) gene expression was increased 1.62-fold. Immune activation was identified as the category with the most changes (containing 60 Gene Ontology (GO) term biological processes (BPs), 41 genes) via functional clustering analysis of altered gene expression. Ge-132 affected genes in clusters related to ATP production (22 GO term BPs, 21 genes), lipid metabolism (4 GO term BPs, 38 genes) and apoptosis (5 GO term BPs). Many GO term BPs containing these categories were significantly affected by the Ge-132 intake. Oral Ge-132 intake may therefore have increased plasma α-tocopherol levels by up-regulating α-tocopherol transfer protein (Ttpa) gene expression.

Key words: organic germanium, tocopherol transfer protein, gene expression, microarray, immune modulation

Introduction

Poly-*trans*-[(2-carboxyethyl) germasesquioxane] (Ge-132) is a water-soluble organic germanium compound that was first synthesized in 1967 by Dr. Hiroshi Oikawa, a professor of Toyo University. Studies have confirmed the structure [1] and safety of this compound in some types of animals [2, 3]. Because it is a polymer, expressed as $(GeCH_2CH_2COOH)_{2n}O_{3n}$, Ge-132 is stable when exposed to certain environmental chemical and physical stresses; but when dissolved in water the Ge-O-Ge bond is broken, and the compound is hydrolyzed to its monomeric form, 3-(trihydroxygermyl) propanoic acid, which is expressed as $(HO)_3GeCH_2CH_2COOH$. The oral intake of Ge-132 has antitumor effects via mediating interferon-induced immune responses [2] and activates leukocytes such as macrophages, natural killer (NK) cells [4] and T cells [5]. The oral administration of Ge-132 also increases interferon-gamma (IFN-γ) levels in the plasma of rodents [4] and humans [2]. Ge-132 is thought to enhance the immune response via the IFN-γ pathway.

In a distributional study of oral Ge-132 administration [6], approximately 20 % of the Ge-132 was absorbed, and quickly excreted from the kidneys into the urine (24 to 48 hours). Recently, we developed a microanalysis method for Ge-132 using an LC-MS/MS system [7]. In a pharmacokinetic study of the report in rats using that system, almost none of the administered Ge-132 had been metabolized to other structures from the monomeric form of Ge-132. Many beneficial properties of Ge-132 have been reported, including anti-rheumatism [8], osteoporosis prevention [9], pain alleviation, and protection against tissue damage. Recently, we reported that Ge-132 causes an increase in Ugt1a1 gene expression; Ugt1a1 encodes uridine diphosphate glucuronosyltransferase isozyme 1a1, which is involved in phase II detoxification in the liver [10]. Because this enzyme catalyzes the conversion of bilirubin to its glucuronosyl conjugate for detoxification and secretion into the bile, enhanced Ugt1a1 expression might result in increased bilirubin excretion in feces [11]. The entire mechanism of dietary Ge-132 is not yet well understood in the liver, however.

Recently, DNA microarray technology has been used to screen for unknown food functions and to clarify the mechanisms of food functionalities [12, 13]. In this study, we examined the effect of dietary Ge-132 intake on hepatic gene expression using microarray technology. We mainly focused on genes that are involved in both bilirubin metabolism and antioxidant induction. We evaluated two antioxidative plasma indices: thiobarbituric acid reactive substances (TBARS) and α- and β-tocopherol.

Materials and Methods

Animals

Male imprinting control region (ICR) mice (ten weeks old; Clea Japan, Tokyo, Japan) were maintained under controlled temperature (22 – 23 °C) and light (08:00 to 20:00) conditions. After acclimation for one week, the mice were randomly divided into three groups (7 mice each) and maintained on a commercial diet (D10001, Research Diets, Inc., New Brunswick, NJ, USA) based on the American Institute of Nutrition (AIN)-76 diet. The three groups were labeled as the Control Group, Day 1 Group and Day 4 Group. The mice in the control group were fed a control diet based on the general AIN-76 composition. Ge-132 was added at 0.05 % of the basal diet because a previous study reported that the dose affects fecal color due to the pigment which having anti-oxidative bilirubin of bile excreted from the liver [10]. To prepare the Ge-132-containing diet, 0.05 % Ge-132 was added to the control diet. The Day 1 Group was fed the control diet for three days and then given the 0.05 % Ge-132-containing diet on the last day. The Day 4 Group was fed the 0.05 % Ge-132-containing diet for four days. On the final day, the food was removed from the cage 4 hours before euthanasia to allow the mice to fast. The mice were anesthetized using diethyl ether and euthanized by bleeding from the cranial mesenteric vein. The liver was then collected and dipped in RNAlater solution (Applied Biosystems Japan, Tokyo, Japan), frozen at –20 °C and stored until RNA extraction was performed.

Evaluation of antioxidative factors in blood plasma by HPLC analyses

The blood samples were centrifuged at 1,900 × g for 15 minutes at 4 °C. The plasma was then collected and stored at –80 °C, and TBARS and tocopherols were identified using high-performance liquid chromatography (HPLC). The methods are described in detail below.

Tocopherol analysis

First, 20 μl of blood plasma was collected in a test tube, and 800 μl of 1 % sodium chloride (w/v) solution and 1 ml of 0.3 μg/ml PMC (2,2,5,7,8-pentamethyl-6-hydroxychroman) in ethanol were added and mixed well. Three milliliters of n-hexane was added, and the mixture was extracted by vortexing for 1 minute. The sample was centrifuged for 10 min at 600 × g, and the upper phase was then transferred to a new tube. The solvent was vaporized with nitride gas to dryness, and the sample was then redissolved in 3 ml of methanol to prepare an HPLC sample. The sample (50 μl) was injected into a reverse phase ODS-100Z column (4.6 × 250 mm; particle size 5 μm; Tosoh, Tokyo, Japan) operating at 40 °C. Methanol was used as the mobile phase at a flow rate of 1.5 ml/min, and the tocopherols were detected using a fluorescence detector (excitation at 298 nm, emission at 325 nm).

TBARS analysis

Twenty microliters of blood plasma was first collected in a test tube, then, 2 ml of 0.2 % thiobarbituric acid (TBA) and 20 μl of 5 % butyl hydroxytoluene (BHT) in ethanol were added to the sample and mixed well. The sample was reacted by boiling for 45 minutes and then cooled in cold water. TBARS were extracted with 2 ml of n-butanol and strong shaking for 5 minutes. The sample was centrifuged for 15 min at 600 × g, and the upper phase was then transferred to a sample tube and analyzed using HPLC. The sample (20 μl) was injected onto a reverse phase ODS-100 V HPLC column (4.6 × 250 mm; particle size 5 μm; Tosoh, Tokyo, Japan) at room temperature. Acetonitrile (20 %) was used as the mobile phase at a flow rate of 0.8 ml/min, and the assayed compounds were detected using a fluorescence detector (excitation at 515 nm and emission at 553 nm).

DNA microarray analysis

The tissue studied (approximately 50 mg) was homogenized using an SK mill (Tokken, Kashiwa, Japan), and the total RNA was then isolated using the SV total RNA isolation system (Promega, Madison, WI, USA) according to the manufacturer's instructions. The isolated total RNA was then subjected to microarray analysis.

Two micrograms from each of the four total RNA samples from the same group was pooled into a tube representing the group. The samples were then subjected to CodeLink microarray system analysis. Two array hybridizations were performed. The target cRNA labeled with biotin-11-UTP was synthesized using a CodeLink Expression Assay Reagent Kit (GE Health Care, Chalfont St. Giles, Buckinghamshire, U.K.) according to the manufacturer's protocol. Briefly, double-stranded cDNA was synthesized using 2 μg of total RNA as the template. To generate the labeled cRNA target, *in vitro* transcription was conducted using the double-stranded cDNA with the incorporation of biotin-11-UTP (Perkin Elmer, Waltham, Mass., USA). For hybridization, 10 μg of cRNA was fragmented and then diluted with the hybridization buffer included in the Expression Assay Reagent Kit. Biotinylated target cRNAs were hybridized to CodeLink Mouse Uniset 20k Bioarrays (GE Healthcare) and processed according to the CodeLink Expression Bioarray System protocol. Processed arrays were scanned using an ArrayWoRxe Scanner (Applied Precision, Issaquah, Wash., USA), and array images were analyzed using Codelink Expression Analysis v4.0 software (GE Healthcare).

GeneSpring version 7.3.1 (Agilent Technologies, Santa Clara, CA, USA) was used to compare the array data. Per-chip normalization was conducted using the 50th percentile of all measurements as a positive control for each sample. This process was followed by per-gene normalization, for which all of the median samples were normalized to 1.0, and the remaining samples were normalized to the median samples. The functional categorization and clustering of genes whose expression levels were altered by dietary Ge-132 was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics tools (http://david.abcc.ncifcrf.gov/).

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

The mouse hepatic samples were stored in RNAlater at –20ºC and then homogenized using an SK mill (Tokken, Inc., Kashiwa, Japan). The total RNA was isolated using the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Two micrograms of extracted total RNA was used as a template for cDNA synthesis via reverse transcription with an Oligo dT primer and Super Script III (Invitrogen,

Carlsbad, CA, USA). The synthesized cDNA was used for quantitative polymerase chain reaction (qPCR) analysis. Quantitative real-time PCR was used to analyze heme oxygenase 1 (Hmox1), heme oxygenase 2 (Hmox2), cytochrome protein 7a1 (Cyp7a1), cholecystokinin (Cck), and beta-actin (Actb, used as a housekeeping control gene) using the following primer pairs: Hmox1: sense primer 5'-gtc aag cac agg gtg aca ga-3' and anti-sense primer 5'-atc acc tgc agc tcc tca aa-3'; Hmox2: sense primer 5'-gga gca gtc atc ttg gat tgt-3' and anti-sense primer 5'-ttg tgg ctg agt agt ttg tgc t-3'; Cyp7a1: sense primer 5'-aaa cac cat tcc tgc aac ct -3' and anti-sense primer 5'-gag gct gct ttc att gct tc-3'; Cck: sense primer 5'-tgc cga gga cta cga ata cc-3' and anti-sense primer 5'-ggt ctg gga gtc act gaa gg-3'; Actb: sense primer 5'-aag tac ccc att gaa cat ggc a-3' and anti-sense primer 5'-ctg gat ggc tac gta cat ggc t-3'. The housekeeping gene Actb was chosen as a stably expressed gene from confirmatory microarray data. The corresponding cDNA was amplified with SYBR Premix Ex Taq II (Takara Bio, Ohtsu, Japan) using an Opticon 2 instrument (Bio-Rad laboratories, Hercules, CA, USA), which was programmed as follows: 95 °C for 30 sec, followed by 40 cycles of denaturation (95 °C for 5 sec), and then annealing and extension (60 °C for 30 sec). Each expression value was calculated according to the threshold cycle value, and the data was displayed as the expression ratio of each gene to Actb.

Ethics of the animal experimentation

The animal experiments were all conducted at Asai Germanium Research Institute Co., Ltd. according to the guidelines provided by the Ethical Committee of Experimental Care, which are based on public guidelines set by the Japanese Ministry of Education, Culture, Sports, Science and Technology. The studies were individually approved following the ethical guidelines by the judging committee at Asai Germanium Research Institute Co. Ltd.

Statistical analysis

The results are presented as the mean ± standard errors of the means (S.E.M.). The normality of the distribution was assessed using Microsoft Excel statistical functions. The significance level of normality was defined as $\alpha = 0.05/2$. The experimental data confirmed normality, were analyzed by analysis of variance (ANOVA). The differences between groups were detected by Tukey's multiple comparison tests. The statistical significance of all experiments was defined as $p < 0.05$.

Results

Oxidative stress markers in blood plasma

Dietary Ge-132 intake induces the antioxidant bilirubin in the intestine, and so we measured two oxidative stress markers: plasma tocopherol concentrations and TBARS. The plasma concentrations of α- and β-tocopherol are shown in Figure 1 A. The concentration of α-tocopherol was increased in a time-course experiment after the intake of dietary Ge-132; on Days 0, 1 and 4, the concentrations were 6.85, 8.57 and 9.60 (μg/ml), respectively. A significant difference was found between Days 0 and 4 ($p<0.05$). The concentrations of β-tocopherol on Days 0, 1 and 4 were 8.39, 8.96 and 6.87 (μg/ml), respectively.

The levels of TBARS are shown in Figure 1B. The levels of TBARS on Days 0, 1 and 4 were 0.71, 0.86 and 0.73 (nmol/ml), respectively, and no significant differences were detected.

QRT-PCR analysis of hepatic gene expression related to bile component secretion and metabolism

The expression of genes related to bile pigment, bilirubin, and bile acid secretion and metabolism were identified because Ge-132 promotes bile component secretion. Cck, Cyp7a1, Hmox1 and Hmox2 gene expression throughout the experiment relative to the expression observed on Day 0 is shown in Figure 2. Cck increased over the time course from Days 0 to 4, and the relative expression levels on Days 1 and 4 compared with Day 0 were 1.80 and 7.61, respectively. Cyp7a1 also increased, and the relative expression levels in the Day 1 and Day 4 groups were 2.07 and 3.94, respectively. Hmox1 and Hmox2 increased; the Hmox1 expression levels on Days 1 and 4 were 1.16 and 2.36, respectively, whereas the Hmox2 expression levels on Days 1 and 4 were 1.43 and 2.17, respectively. Significant ($p < 0.05$) differences were found for Cck and Hmox2 between Days 0 and 4.

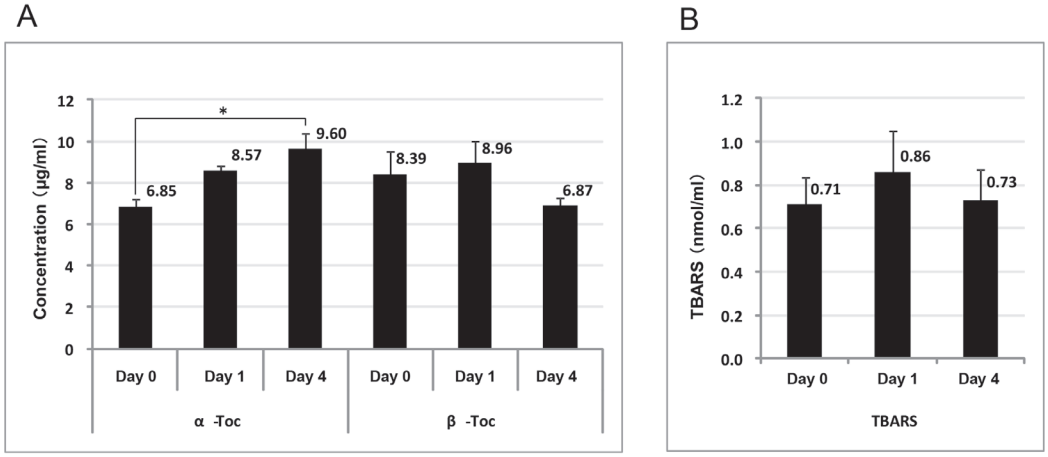

Figure 1: Changes in oxidative markers in blood plasma resulting from dietary Ge-132 intake in mice. Three groups of mice received the 0.05 % Ge-132-containing AIN-76 diet for 0, 1 or 4 days. The levels of alpha- and beta-tocopherol (A) and TBARS (B) were measured in mouse blood plasma using HPLC. Asterisks show significant differences ($p<0.05$) between the groups. Error bars represent the S.E.M. (n=7).

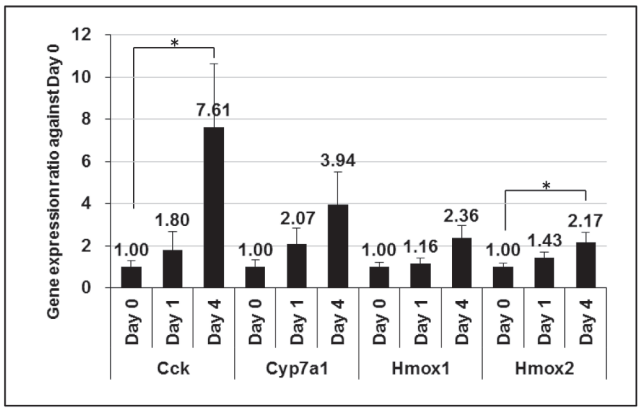

Figure 2: Hepatic gene expression related to bile component metabolism and secretion after dietary Ge-132 intake in mice. Hepatic Cck, Cyp7a1, Hmox1 and Hmox2 gene expression in response to Actb was analyzed using quantitative PCR. The values shown represent the relative expression (compared with that in the Day 0 group). The 0.05 % Ge-132-containing diet was fed to mice for 0, 1 or 4 days. Total RNA was extracted from the livers. Values with asterisks are significantly different between two groups ($p<0.05$). Error bars represent the S.E.M. (n=7).

Microarray analysis

The oral intake of dietary Ge-132 for one day affected gene expression in the livers of the mice. Using GeneSpring software, 10,940 gene probes were identified as being expressed at high levels (raw data signals greater than 50), with data flags of "presence" or "marginal" found in both groups. Numerous genes were up-regulated or down-regulated more than 1.5-fold (relative to the control group) after less than 24 hours of dietary Ge-132 intake. We identified 606 up-regulated genes and 706 down-regulated genes. Expression of the Cck gene NM_031161 was enhanced 8.17-fold, which is an extremely high change among the 606 up-regulated genes. The changes of certain genes related to anti-oxidative functions are shown in Table I. Expression of the Ttpa and glutathione synthase (Gss) genes was increased 1.62- and 1.77-fold, respectively.

A cluster analysis was performed using DAVID bioinformatics tools, which are available on the web (http://david.abcc.ncifcrf.gov/); the 606 up-regulated genes were clustered into Gene Ontology (GO) term groups based on their biological process (BP) and were annotated based on the functional annotation chart using DAVID. Thirty-one clusters were annotated, and the data was filtered based on the modified Fisher's Exact P-value. After filtering, the number of clusters was reduced to 16. The number of BP-annotated GO term clusters in these 16 clusters is shown in Figure 3. The total number of all BP GO term clusters after filtering by P-value was 152. As shown in Figure 3, the largest cluster, which contained 60 terms, is categorized as containing immune activation-related terms. The clusters labeled "purine nucleotide biosynthesis" and "metabolic process and carbohydrate metabolic process" contained 22 and 12 terms, respectively. Clusters related to "lipid homeostasis," "organic acid and amino acid metabolic processes," "antigen processing and presentation," "induction of apoptosis," "DNA damage stimulus" and "ncRNA metabolic process

Table I: Genes coding for antioxidant-related proteins were altered by dietary Ge-132 after one day of intake in the liver of ICR mice.

GenBank accession	Gene symbol	Gene name	Fold change
NM_008180	Gss	Glutathione synthetase	1.77
NM_015767	Ttpa	Alpha-tocopherol transfer protein	1.62
NM_016892	Ccs	Copper chaperone for superoxide dismutase	1.45
NM_008161	Gpx3	Glutathione peroxidase 3	1.33
NM_008160	Gpx1	Glutathione peroxidase 1	1.31
NM_013671	Sod2	Superoxide dismutase 2, mitochondrial	1.31
NM_010343	Gpx5	Glutathione peroxidase 5	0.99
NM_172203	Nox1	NADPH oxidase 1	0.93
NM_009804	Cat	Catalase	0.79

Fold change represents the ratio of the expression of the Ge-132 group compared with that of the control group. The data was obtained from a microarray analysis (no statistical information included).

energy derivation of the TCA cycle" were also found. The clusters obtained for the down-regulated gene groups were small, however, and trends regarding the specific 'GO term BP' terms were not found, except for two small clusters related to proteolysis and primary metabolic processes.

We filtered out the items with significance levels of $p < 0.05$ using the Benjamini method based on the false discovery rate (FDR) [14], and the clusters of up-regulated genes in each 'GO term BP' category are listed in Table II. The largest group was the category related to "immune activation" (annotation cluster 5). This group contained items regarding the innate immune response ("GO:0019884, antigen processing and presentation of exogenous antigen") and adaptive immune responses ("GO:0019724, B-cell-mediated immunity" and "GO:0002250 adaptive immune response"). Indeed, the terms GO:0002478, 0019884 and 0048002 for antigen processing and presentation, related to the innate immune response, and the term GO:0002250, related to the adaptive immune response, included 8 and 15 genes, respectively. Antigen

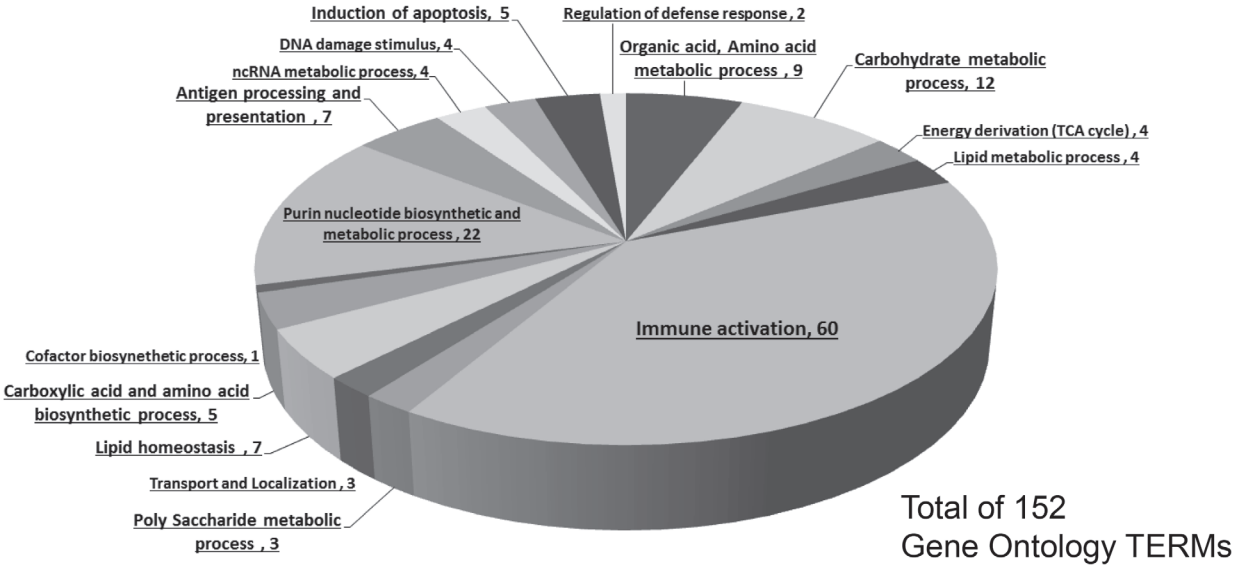

Figure 3: Cluster analysis of hepatic gene expression influenced by dietary Ge-132 intake in mice. The gene expression profile of one day Ge-132 intake was compared with the control group (no Ge-132 intake). The global hepatic gene expression profile was analyzed using microarray technology. A cluster analysis for genes with increased or decreased expression of more than 1.5-fold was performed using DAVID bioinformatics tools (http://david.abcc.ncifcrf.gov/).

Table II: Clusters of gene ontology biological process terms altered by 1-day Ge-132 intake in the livers of mice.

Category	Gene Ontology Biological Process Term	Altered genes	*P*-Value	Benjamini*
Organic acid, amino acid metabolic process -Annotation Cluster 1: Enrichment Score: 6.32				
	GO:0019752 carboxylic acid metabolic process	41	4.5E-09	4.9E-06
	GO:0043436 oxoacid metabolic process	41	4.5E-09	4.9E-06
	GO:0006082 organic acid metabolic process	41	4.7E-09	3.5E-06
	GO:0042180 cellular ketone metabolic process	41	9.1E-09	5.0E-06
	GO:0009308 amine metabolic process	30	3.3E-07	8.0E-05
	GO:0006520 cellular amino acid metabolic process	20	4.7E-06	6.9E-04
	GO:0044106 cellular amine metabolic process	23	9.4E-06	1.3E-03
	GO:0006519 cellular amino acid and derivative metabolic process	24	3.2E-05	3.3E-03
Carbohydrate metabolic process -Annotation Cluster 2: Enrichment Score: 3.59				
	GO:0005975 carbohydrate metabolic process	35	8.8E-07	1.8E-04
	GO:0005976 polysaccharide metabolic process	13	1.9E-05	2.1E-03
	GO:0044262 cellular carbohydrate metabolic process	24	1.4E-04	9.2E-03
	GO:0006066 alcohol metabolic process	25	2.2E-04	1.4E-02
	GO:0006006 glucose metabolic process	14	3.0E-04	1.7E-02
	GO:0044042 glucan metabolic process	7	3.9E-04	2.2E-02
	GO:0005977 glycogen metabolic process	7	3.9E-04	2.2E-02
	GO:0006073 cellular glucan metabolic process	7	3.9E-04	2.2E-02
	GO:0019318 hexose metabolic process	15	5.7E-04	2.7E-02
	GO:0006112 energy reserve metabolic process	7	7.4E-04	3.3E-02
Energy derivation (TCA cycle) -Annotation Cluster 3: Enrichment Score: 3.45				
	GO:0015980 energy derivation by oxidation of organic compounds	16	2.1E-07	6.5E-05
	GO:0006084 acetyl-CoA metabolic process	8	2.9E-05	3.2E-03
	GO:0006732 coenzyme metabolic process	15	1.0E-04	7.5E-03
	GO:0051186 cofactor metabolic process	17	1.2E-04	8.3E-03
Lipid metabolic process -Annotation Cluster 4: Enrichment Score: 2.77				
	GO:0006629 lipid metabolic process	38	5.1E-04	2.6E-02
Immuno-activation -Annotation Cluster 5: Enrichment Score: 2.73				
	GO:0002526 acute inflammatory response	15	1.1E-07	4.9E-05
	GO:0002250 adaptive immune response	15	1.8E-07	6.5E-05
	GO:0002460 adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	15	1.8E-07	6.5E-05
	GO:0016064 immunoglobulin-mediated immune response	13	3.0E-07	8.2E-05
	GO:0019724 B cell-mediated immunity	13	4.3E-07	9.4E-05
	GO:0002449 lymphocyte-mediated immunity	13	2.4E-06	4.1E-04
	GO:0002478 antigen processing and presentation of exogenous peptide antigen	8	3.3E-06	5.2E-04
	GO:0002443 leukocyte-mediated immunity	13	1.3E-05	1.7E-03

Category	Gene Ontology Biological Process Term	Altered genes	*P*-Value	Benjamini*
	GO:0019884 antigen processing and presentation of exogenous antigen	8	1.4E-05	1.7E-03
	GO:0006950 response to stress	61	3.7E-05	3.6E-03
	GO:0009605 response to external stimulus	39	4.6E-05	4.4E-03
	GO:0006954 inflammatory response	20	4.8E-05	4.4E-03
	GO:0048002 antigen processing and presentation of peptide antigen	8	6.7E-05	5.6E-03
	GO:0050764 regulation of phagocytosis	7	7.6E-05	6.0E-03
	GO:0002252 immune effector process	14	1.0E-04	7.5E-03
	GO:0009611 response to wounding	25	1.3E-04	8.8E-03
	GO:0006956 complement activation	7	6.3E-04	2.9E-02
Polysaccharide metabolic process -Annotation Cluster 7: Enrichment Score: 2.43				
	GO:0005976 polysaccharide metabolic process	13	1.9E-05	2.1E-03
Purine nucleotide biosynthetic and metabolic process -Annotation Cluster 13: Enrichment Score: 1.54				
	GO:0044271 nitrogen compound biosynthetic process	21	8.0E-04	3.4E-02
Antigen processing and presentation -Annotation Cluster 14: Enrichment Score: 1.47				
	GO:0002478 antigen processing and presentation of exogenous peptide antigen	8	3.3E-06	5.2E-04
	GO:0019884 antigen processing and presentation of exogenous antigen	8	1.4E-05	1.7E-03
	GO:0048002 antigen processing and presentation of peptide antigen	8	6.7E-05	5.6E-03
	GO:0002495 antigen processing and presentation of peptide antigen via MHC class II	5	1.1E-03	4.2E-02
	GO:0019886 antigen processing and presentation of exogenous peptide antigen via MHC class II	5	1.1E-03	4.2E-02
	GO:0019882 antigen processing and presentation	10	1.2E-03	4.4E-02

The data shows the functional annotation clustering of 606 increasingly altered genes by Ge-132. The DAVID bioinformatics database (http://david.abcc.ncifcrf.gov/) was used for functional gene classification.
The gene ontologies were filtered by the Benjamini method at a statistical significance of $p<0.05$.
The p-value is defined by a modified Fisher's exact test.
* The Benjamini method controls the false discovery rate based on Benjamini and Hochberg [14]

processing and presentation were observed in an independent cluster that included 6 GO BP terms describing antigen processing and presentation. This cluster included the two terms GO:0002495 and GO:0019886, which are related to major histocompatibility complex (MHC) class II molecules. Incidentally, annotation clusters 1, 2 and 4 were related to the metabolism of organic acids, carbohydrates and lipids, respectively. Energy metabolism based on the tricarboxylic acid (TCA) cycle was also found in these clusters.

Figure 3 and Table II suggest that immune activation and antigen presentation are important clusters. The changes in mRNA expression of hepatic Fc receptors after 1 day of Ge-132 intake were examined from DNA microarray analysis data and are shown in Table III. Seven Fc receptor genes were detected using this DNA microarray analysis, and 4 genes (Fcgr2b, Fcer1 g, Fcgr1 and Fcgr3) were increased by 2.53-, 2.31-, 2.03- and 1.51-fold, respectively, after Ge-132 intake.

Discussion

Ge-132 is a synthetic water-soluble organic germanium compound that has been found to be safe (LD_{50} estimated 11,700 mg/kg for rat oral administration [15], LD_{50} > 125,250 mg/kg for dog intravenous administration [16]) and is currently used in foods and cosmetics in Japan, Korea, China and the U.S.A. In Japan, the Ministry of Health, Labor and Welfare announced that the safety germanium in food should

Table III: Effects of 1-day Ge-132 intake on the mRNA level of hepatic Fc receptors in mice.

GenBank accession	Gene symbol	Gene name	Fold change	Related KEGG pathways
NM_010187	Fcgr2b	CD32; Fcgr2; FcgRII; Fcgr2a; Ly-m20	2.53	KEGG pathway: B cell receptor signaling pathway 04662
NM_010185	Fcer1g	CD23; Fce1g; Ly-50; FcR-gamma	2.31	KEGG pathway: Fc epsilon RI signaling pathway 04664 KEGG pathway: Natural killer cell-mediated cytotoxicity 04650
NM_010186	Fcgr1	CD64; FcgammaRI	2.03	KEGG pathway: Hematopoietic cell lineage 04640
NM_010188	Fcgr3	CD16	1.51	KEGG pathway: Natural killer cell-mediated cytotoxicity 04650
NM_144559	Fcgr3a	Fcrl3; CD16-2; FcgRIV; FcgammaRIV	1.13	KEGG pathway: Natural killer cell-mediated cytotoxicity 04650
NM_010189	Fcgrt	FcRn	0.83	–
NM_013517	Fcer2a	CD23; Fce2; Ly-42	0.77	KEGG pathway: Hematopoietic cell lineage 04640
NM_144960	Fcamr	MGC129330; MGC129331	0.76	–

Fold change represents the expression ratio of the Ge-132-fed group compared with the control group (no statistical information included).
KEGG: Kyoto Encyclopedia of Genes and Genomes database (http://www.genome.jp/kegg/).

be confirmed because the toxicity of some germanium compounds caused fetal accidents [17]. For example, germanium dioxide has physiological hematopoietic activity [18]; however, germanium overdose triggers kidney breakdown. In Europe, some fatal accidents using lactate-citrate germanium have been reported. The lactate-citrate germanium is not organic germanium, however, but is a complex between organic acid and a toxic germanium dioxide [19]. In reference to the safety of Ge-132, Sanai et al reported that germanium dioxide is toxic [20]. Understanding the structure and confirming the safety of germanium as a food component is essential. Ge-132 is a crystal polymer of (3-trihydroxygermyl) propanoic acid. The crystal structural of the compound was confirmed, and Ge-132 was developed for medical use as an anti-cancer drug to mediate immune modulation. Ge-132 could not be cleared through clinical tests to permit the use of the novel drug. Another form of the polymer (3-trihydroxygermyl) propanoic acid (called 3-oxygermylpropinonic acid), propagermanium, is permitted for drug use in chronic hepatitis as a chemokine receptor 2 inhibitor [21]. The crystal structure of propagermanium has not been confirmed, but its IR analytical data differs from Ge-132. Recently, an inhibitory effect of propagermanium against cancer metastasis has been reported [22].

As previously described by our group, Ge-132 enhances the secretion of bile components and antioxidative pigments such as bilirubin and urobilinogen [10], and we therefore evaluated blood plasma antioxidative markers in this study. Additionally, we evaluated the influence of dietary Ge-132 intake on hepatic gene expression using DNA microarray technology.

Dietary Ge-132 intake increased the plasma α-tocopherol levels; however, the TBARS levels were unchanged. Tocopherols are fat-soluble antioxidant vitamins, and α-tocopherol (vitamin E) is well known to have a high antioxidative capacity. Ge-132 intake might have increased α-tocopherol via the suppression of oxidative stress through the induction of other antioxidants such as bilirubin and urobilinogen. Previously, we reported that the radical-trapping activities of bilirubin and urobilinogen for the stable radical DPPH (1,1-diphenyl-2-picrylhydradyl) are higher than that of α-tocopherol [23]. Urobilinogen and bilirubin, which are induced by Ge-132 intake, might therefore be used prior to α-tocopherol. According to the microarray analysis data, however, Ttpa, which encodes tocopherol transfer protein, was up-regulated by Ge-132 intake. Ttpa selectively transfers α-tocopherol from hepatocytes to the blood plasma [24, 25], and therefore, Ge-132 intake might increase α-tocopherol levels in the plasma by inducing tocopherol transfer protein expression.

We observed interesting changes in the expression of genes involved in bile secretion. We previously demonstrated that bile secretion is enhanced by dietary Ge-132 intake [10]. Cck is a hormone that

plays a role in bile secretion; additionally, a new role for Cck as an anti-inflammatory factor has been revealed [26–28]. Our analysis of the microarray and RT-qPCR data showed that the hepatic expression of Cck was increased to high levels after oral Ge-132 intake. Bile secretion can be enhanced by Ge-132 intake via an increase in the expression levels of Cck. The hormone Cck is generally secreted from the duodenum, however, if Cck is secreted from the liver, it must exert a stronger effect than the secretions from the duodenum because the Cck target organs such as the gallbladder and pancreas are located next to the liver. A detailed study related to the up-regulation of Cck secretion from the liver is expected. Cyp7a1 catalyzes the conversion of cholesterol to bile acid [29]; as we described in a previous report, the secretion of bile pigment, bilirubin and total bile acid is enhanced by Ge-132 [10], and the increased Cyp7a1 expression is consistent with the increased total bile acid secretion. Bilirubin is a metabolite of hemin, and Hmox catalyzes ring opening of the porphyrin structure in hemin to convert it to biliverdin, which is then reduced to bilirubin [30]. In this study, Hmox1 and Hmox2 expression was increased by oral Ge-132 intake in a time-dependent manner, which might explain why bilirubin is increased in feces. Hmox1 is a stress protein, and its expression is induced by various stimulating factors [31, 32], but Hmox2 is constitutively expressed at a constant level [30]. Cobalt chloride induces Hmox2 expression [33], and Ge-132 might induce Hmox2 expression in a similar manner. Hmox produces ferric ions, biliverdin and carbon monoxide (CO); additionally, Hmox1 and the CO it releases are therapeutic targets for liver injury [34]. Dietary Ge-132 might protect against liver injury by inducing hepatic CO production. As we have previously reported, bilirubin and its reduced metabolite, urobilinogen, are antioxidants [23, 10, 35]. We thus examined tocopherol and TBARS levels in the blood plasma for use as indices of antioxidative capacity. Oral Ge-132 intake increased α-tocopherol in a time-dependent manner up to Day 4, however, TBARS levels did not change during Ge-132 administration. These findings suggest that α-tocopherol increased because bilirubin and urobilinogen were increased by Ge-132, and bilirubin and urobilinogen are mainly antioxidants that are released prior to α-tocopherol. This experiment was not conducted under oxidative stress conditions but rather under normal conditions, and therefore, the TBARS levels in the control group (Day 0 of Ge-132 administration) might not be sufficiently high to evaluate the suppression of oxidative stress by Ge-132 intake because the normal potency of various anti-oxidative factors to reduce oxidative stress may be high enough in mice under normal conditions.

The short-term oral administration of Ge-132 as a dietary component changed the expression of many genes. In many cases, up-regulated genes were categorized as GO BP term categories using DAVID cluster analyses. The immune response group was the largest group of genes; the expression of these genes was altered by Ge-132 intake (Figure 3 and Table II), and the group contained 60 GO BP term categories for immune activation (Figure 3). The total number of hit terms was 152; therefore, this group represented 39.5 % of all hits. Ge-132 has previously been used as an immune modulator [2], and numerous studies have reported its effects on the immune response [4, 36–39], including IFN-γ induction, macrophage and natural killer cell activation. Antitumor effects of Ge-132 have also been found in animal studies [2]. In this study, 10 genes that play roles in antigen processing and presentation (GO:0019882) were up-regulated (Table II). Previous studies have shown that Ge-132 activates macrophages, and this study evaluated hepatic gene expression; therefore, Kupffer cells might have been activated. Many dendritic cells (DCs) are present in the liver [40–42], however, and these cells play a role in MHC class II antigen presentation. The data related to GO:0002495 and GO:0019886 might indicate that Kupffer cells and/or DCs were activated by Ge-132. The innate immune response is stimulated by Ge-132, which enables phagocytosis by Kupffer cells and DCs to proceed. Kurane et al. revealed that splenic cell Fc receptors were increased or decreased by Ge-132 administration [43]. In this study, the expression of four Fc receptors was higher in the group fed a diet with Ge-132 (Table III). The Fc receptor is important for antigen presentation [44–46]. In this study, both the immunoglobulin-mediated response (GO:0016064) and B-cell-mediated immunity (GO:0019724) categories contained 13 up-regulated genes; therefore, Ge-132 might stimulate adaptive immune system-related leukocytes at an early stage after administration. In a previous study, Suzuki suggested that leukocytes are activated by Ge-132 [36, 5, 47]; therefore, the data obtained here might be related to the data reported by Suzuki. Recently, we reported that Ge-132 cooperates with lactobacilli and oligosaccharides to activate intestinal immunity [39]. Stimulation by intestinal microorganisms might enhance the immune activation caused by Ge-132 via antigen-presenting cells.

We also found increased α-tocopherol levels in the plasma and bilirubin induction in the bile by Ge-132. These antioxidants protect the body from oxidative stress in a homeostatic manner. Generally, oxidative stress suppresses immunity, thereby causing disease [48, 49]. Ge-132 might protect the immune system from

oxidative stress by increasing endogenous antioxidants such as α-tocopherol, bilirubin and urobilinogen.

The immune system was not the only defense response noted. The complement factor group included hits found using the DAVID analysis (Figure 3). Complement factor, which represents the earliest defense factor, was up-regulated. This result was obtained using microarray analysis in the group that received the Ge-132-containing diet for only one day. Ge-132 may therefore induce a total defense response in humans at an early stage after intake.

In our next study, we intend to evaluate the effects of Ge-132 administration over a longer period of up to several weeks. The present study was conducted over a very short period of Ge-132 intake, and we must evaluate continuous Ge-132 use as a dietary supplement for health care.

In this study, microarray analysis revealed the up-regulation of GO terms related to DNA damage and ncRNA metabolism (Figure 3). Ge-132 might protect DNA from damage caused by any stress. Mochizuki and Kada revealed that Ge-132 administration produces an anti-mutagenic effect that is caused by the prevention of DNA damage by gamma radiation [50]. Similar results were obtained using animal studies (unpublished data). The microarray analysis data supports these previous results.

Conclusion

In this study, we evaluated the effect of dietary Ge-132 intake on plasma antioxidative markers and hepatic gene expression using microarray analysis. Dietary Ge-132 intake induced Ttpa gene expression in the liver and increased α-tocopherol levels in the plasma. The nutrigenomics method applied here is also useful for evaluating compounds such as Ge-132 that possess various functions. The results of this study were consistent with those found in many previous studies, and new findings on gene expression were made. Dietary Ge-132 intake was shown to induce various antioxidative effects that might protect the immune system from oxidative damage. We expect this gene profiling technique to be applied to blood samples from humans taking Ge-132 supplements.

Acknowledgements

We are grateful to Miki Saito of the Research Division at the Asai Germanium Research Institute for technical assistance with the analyses of antioxidative factors.

Tomoya Takeda analyzed part of the data in the microarray analyses; Yoshihiko Tokuji performed the microarray analysis experiment; Takashi Nakamura designed the study and performed the experiments, analyzed the data, and wrote the manuscript.

Conflicts of interest

Takashi Nakamura and Tomoya Takeda are employed by the Asai Germanium Research Institute Co., Ltd. We did not receive any outside funding or grants for this study.

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