Nutrigenomic studies of effects of Chlorella on subjects with high-risk factors for lifestyle-related disease

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ABSTRACT In order to clarify the physiological effects of Chlorella intake on subjects with high-risk factors for lifestyle-related diseases, we conducted Chlorella ingestion tests on 17 subjects with high-risk factors for lifestyle-related diseases and 17 healthy subjects over a 16-week period, including a 4-week post-observation period. We conducted blood biochemical tests and analyzed gene expression profile in whole blood cells in the peripheral blood before and after Chlorella intake. We confirmed that in both groups, Chlorella intake resulted in noticeable reductions in body fat percentage, serum total cholesterol, and fasting blood glucose levels. Through gene expression analysis, we found that gene expression profiles varied with Chlorella intake and identified many genes that exhibited behavior such that after the completion of the intake period, expression levels returned to pre-intake expression ones. Among these were genes related to signal transduction molecules, metabolic enzymes, receptors, transporters, and cytokines. A difference in expression level was found between the two groups at the start of the tests, and we were able to identify genes with noticeable variance in expression level resulting from Chlorella intake in the high-risk factor group. These included genes involved in fat metabolism and insulin signaling pathways, which suggests that these pathways could be physiologically affected by Chlorella intake. There were clear variations in the expression profiles of genes directly related to uptake of glucose resulting from Chlorella intake, indicating that the activation of insulin signaling pathways could be the reason for the hypoglycemic effects of Chlorella.

KEY WORDS: * hypocholesterolemic effect * hypoglycemic effect * insulin * insulin signaling pathway * gene expression * microarray * peripheral blood

INTRODUCTION

CLORELLA HAS BEEN CONSUMED by humans as a food supplement for generations because it is rich in essential nutrients, including high-quality protein, vitamins, minerals, and essential amino acids. In recent years, it has gained attention as a health food because it offers an outstanding balance of nutritional elements. Notably, it has been reported that Chlorella demonstrates physiological effects such as immune activation, (1) growth promotion, (2) and improvement in stress-related ulcers. (3) Most of this prior research, however, used rats, mice, and other laboratory animals; almost no large-scale clinical research has been undertaken to evaluate its physiological effects in humans.

The physiological effects of specific foods have long been known empirically, but because foods are composed of diverse components, it has been difficult to identify the components that are effective in bringing about desirable physiological effects. At the same time, there has been little research conducted on the mechanisms of these physiological effects, because of the lack of effective testing and analytical methods that take into account multiple aspects of these physiological effects. Amid a growing demand for health foods in recent years, there have been calls for proof of the effectiveness of these foods in humans, and for clarification of the mechanisms involved. The term "evidence-based food" is representative of this trend. (4)

Since the announcement in 2004 that the entire human genome sequence had been decoded, attention has been focused on a new research field called "nutrigenomics," which attempts to examine the utility of food products based on genomic information. (5) Specifically, a DNA microarray that can examine expression level of several thousand to several tens of thousands of genes in a single assay has come to be used extensively as a new tool for investigating diverse reactions in living organisms. This microarray technology is extremely well suited to comprehensive examinations of complex reactions in living organisms in which multiple components bring about complex effects, as in the case when food is ingested.

In this research, we analyzed the gene expression profiles in all peripheral blood cells following Chlorella intake in 17 subjects with high risk factors for lifestyle-related diseases (diabetes or hyperlipemia) and 17 healthy subjects. We also gathered biochemical test data from these subjects.

As a result of these tests, we confirmed that in both groups, Chlorella intake resulted in noticeable reductions in body fat percentage, total blood serum cholesterol, and fasting blood glucose levels. Through gene expression analysis, we found that gene expression varied with Chlorella intake and identified many genes whose expression returned to pre-intake expression levels after the completion of the intake period. In the results for insulin signaling pathways in particular, variations were observed in the gene clusters directly related to active uptake of glucose, which provides evidence for the blood glucose reduction effects of Chlorella.
MATERIALS AND METHODS

Subjects

The subjects were healthy Japanese males 20 years of age or older. On three occasions—6 weeks, 4 weeks, and 2 weeks—before the start of the ingestion tests, the subjects were examined by a physician and also received physical examinations, clinical examinations, and glucose tolerance tests. Based on the conditions outlined below, 34 subjects were selected, with 17 assigned to each of two groups: the high-risk factor (for lifestyle-related diseases) group (referred to here as the “D Group”) and the normal healthy subject group (referred to here as the “N Group”).

Healthy subject group (N Group). At all three periods before beginning the tests, fasting blood glucose, total blood serum cholesterol, and concentration of triglycerides in the blood were within normal limits. These subjects were also judged as having normal glucose tolerance.

High-risk factor group (D Group). Subjects who at all three periods before beginning the tests (a) exhibited borderline high fasting blood glucose and total blood serum cholesterol and high triglycerides in blood and who were also judged as having low glucose tolerance or (b) demonstrated borderline high fasting blood glucose, were judged as having low glucose tolerance, and were also judged to have total blood serum cholesterol and serum triglycerides that deviated slightly from normal limits. Attributes for subjects in the N Group and D Group are summarized in Table 1. Serum was used for biochemical tests. Body fat percentage was evaluated by measuring bioelectrical impedance.

Materials tested

The Chlorella used in these tests was “Sun Chlorella A” tablets (Sun Chlorella Corp., Kyoto, Japan), which contains dried Chlorella powder (more than 95.5%) as the active ingredient and lecithin (less than 4.5%) as a bulking agent. The Chlorella powder contained in the tablet was prepared by crushing the cell wall in a DYNO[R]-Mill (WAB, Inc., Basel, Switzerland) and spray-drying. The subjects took 20 tablets each morning and evening (total, 40 tablets/day) after meals with either cold or hot water.

Test design

This research protocol was approved by the Testing Committee at Miyawaki Orthopedic Hospital (Hokkaido, Japan). Before the tests began, the subjects received a written explanation and consent form from the physicians responsible for the tests. After receiving an explanation of the purpose and value of the tests as well as the methods, expected effects, and potential risks, etc., the subjects themselves confirmed their understanding of the details explained and then provided written consent indicating that they were participating of their own free will.

The tests were conducted in an open test format. The intake period lasted for 12 weeks; blood biochemical tests were conducted every 4 weeks, and again 4 weeks after the completion of the intake period.

During the test period, one subject from the N Group dropped out because of stomach pains, but no other subjects dropped out during the testing period. Data for statistical analysis were thus gathered for 17 subjects in the D Group and 16 subjects in the N Group. None of the subjects in either group reported any complications that could be considered to indicate harmful side effects during any of the four physical examinations conducted during the testing period.

[FIGURE 1 OMITTED]

Gene expression analysis

The blood sampling and RNA extraction for gene expression analysis was conducted using a PAXgene[TM] Blood RNA kit (manufactured by PreAnalytiX GmbH, Hombrechtikon, Switzerland), and a model 2100 Bioanalyzer (manufactured by Agilent Technologies, Palo Alto, CA) was used to confirm that there was no breakdown in the extracted RNA (rRNA profile used as a reference). RNA amplification reactions were checked based on the in vitro transcription method using primers with T7 promoter sequence. At this time, cRNA was synthesized through the uptake of dUTP with an aminoallyl group. The cRNA with Cy5 labeling was synthesized by applying coupling reactions to cRNA and Cy5 with a succinimide group (manufactured by GE Healthcare, Chalfont St. Giles, UK).

As the control sample for expression analysis, we used total RNA from commercially available human white blood cells (from Clontech Laboratories, Palo Alto), and Cy3 was used as a fluorochrome. In addition, we synthesized cRNA with the same labeling as the above blood-derived sample and used this cRNA as a common comparative reference sample for all blood-derived samples.

For the microarray, we used a custom microarray with additional loading of diabetes-related genes and other genes on a human drug response DNA chip (manufactured by Hitachi Ltd., Saitama, Japan) (number of genes loaded, 1,873). After the labeled cRNA is mounted on the microarray, it is subject to competitive hybridization at 45[degrees]C for 17 hours. Once hybridization is complete, the unit is washed and dried, and fluorescent images are captured using a scanner (ScanArray5000, GSI Lumonics, Billerica, MA). Through numerical processing, we then derived the variable ratio for expression intensities between the samples and the reference samples for each of the relevant genes. The global normalization method was used for normalization of Cy5 and Cy3.
RESULTS

Blood analysis

Figure 1 shows changes over time in fasting blood glucose, body fat percentage, total blood serum cholesterol, high-density lipoprotein (HDL)-cholesterol, and low-density lipoprotein (LDL)-cholesterol. Each of the measured values shows decreasing trends after Chlorella intake and increasing trends after completion of the intake test period (12 weeks). The measured values before the start of intake (0 weeks) and at each test point were tested for significance using paired t tests. Using a significance level of 5%, both the N Group and the D Group demonstrated significant differences in body fat percentage and total blood serum cholesterol from weeks 4 to 12. Particularly in the case of the D Group, decreases in HDL-cholesterol and LDL-cholesterol showed clearly significant differences at all measurement points after the start of Chlorella intake. Blood glucose levels were also significantly decreased in the D Group after 8 weeks of chlorella consumption. In all of these measured values, a temporary decreasing trend could be seen after the start of Chlorella intake; after the completion of the intake period, these values returned to the levels before intake began. This indicates that the variations in blood parameters were brought about as a result of Chlorella intake. On the other hand, no clear variations in volumes of triglycerides in the blood were observed before and after the start of Chlorella intake in either the healthy subject group or the high-risk factor group. Furthermore, no trends toward increased concentration of insulin in the blood could be seen as a result of Chlorella intake.

Based on the above data, we have summarized the effects of Chlorella intake as follows. With regard to fat metabolism, although no significant changes could be seen in the concentration of triglycerides in the blood, both body fat percentage and total serum cholesterol were decreased in both the N and D Groups. With regard to glucose metabolism, however, Chlorella lowered blood glucose in the D Group. Since no clear increases in insulin concentrations in the blood were observed, the decrease in serum glucose concentrations may be due to improved insulin sensitivity induced by Chlorella intake.

Gene expression analysis

In both N and D Groups, we identified the genes whose mRNA expression levels varied owing to Chlorella intake by comparing expression intensities at 0 weeks (before the start of intake tests) and 4 weeks and between 0 weeks and 12 weeks. Moreover, the genes whose mRNA expression levels exhibited behavior consistent with the blood chemistry data, namely, gene expression profile varied with Chlorella intake and then returned nearly to pre-intake expression levels, were extracted by comparison between expression change of 4 weeks versus 0 weeks and that of 16 weeks versus 0 weeks based on t test. For these identifications, we used t tests with the False Discovery Rate = 0.05 as the level of significance. (6) Next, the genes superimposed in these two kinds of identified genes were extracted in both the N and D Groups. A total of 129 genes were chosen, 65 of which are associated with canonical pathways in the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg) database and are listed in Table 2. Many kinds of genes involved in signal transduction, metabolism, receptors, transporters, and cytokines were included. Moreover, this result suggested that many kinds of pathways involved in the insulin signaling pathway and immunological function may be influenced by Chlorella intake.

In the D Group, significant differences resulting from Chlorella intake were observed for fasting blood glucose, body fat percentage, and total serum cholesterol. In order to identify the genes associated with these physiological effects, we extracted genes that demonstrated differences in expression level between the D Group and the N Group before the start of the Chlorella intake tests and that also varied as a result of Chlorella intake. Table 3 lists the 18 genes thus identified. When we referred to the KEGG database to determine which pathways the identified genes were associated with, we found two genes (protein tyrosine phosphatase 1B and growth factor receptor-bound protein 2) that are associated with the insulin signaling pathway. We then investigated the changes in expressions for the genes among the loaded genes that were associated with the insulin signaling pathway. The results of this investigation are shown in Figure 2, which shows that after Chlorella intake, there is an increase in expression levels for genes related to the signal transduction routes linked to translocation of glucose transporter (GLUT4) below the insulin receptors (producing insulin receptor substrate, phosphoinositol-3-kinase, 3-phosphoinositide-dependent kinase-1, and v-akt murine thymoma viral oncogene homolog 3). Protein tyrosine phosphate-1B (PTP1B) acts to suppress signal transduction, but the expression level for PTP1B showed a tendency to decrease as a result of Chlorella intake. Based on the changes in gene expression levels, we can therefore surmise that insulin signaling pathways are activated by the intake of Chlorella. Recently, Cheng et al. (7) reported that in in vitro screening systems using monocytes from human peripheral blood, Chlorella inhibited the activation of PTP1B.

DISCUSSION

Cherng and Shih (8) reported changes in blood glucose concentrations resulting from administration of Chlorella in streptozotocin-induced diabetic mice. In that study, they reported that administration of Chlorella (100 mg/kg) steadily reduced both glucose concentrations in the blood and increased glucose values during glucose tolerance tests, but that no increases in insulin concentrations in the blood could be seen. Their results correspond closely with the results of the current research on humans. It has also been reported that in streptozotocin-diabetic mice, Chlorella intake increases glucose uptake in the liver and skeletal muscles. (9)

Dimitriadis et al. (10) reported that in monocyte in vitro systems separated from peripheral blood, insulin exposure brought about an increase in the uptake of glucose and a translocation of GLUT4 to the membrane surface. Estrada et al. (11) reported that exposing peripheral blood monocytes from healthy subjects and insulin-dependent diabetic patients to insulin-like growth factor-I immediately causes uptake of glucose and that although the relationship of insulin-like growth factor-I concentration and glucose uptake volumes was similar in both healthy subjects and diabetes patients, the cells obtained from diabetic patients demonstrated lower uptake volumes overall. These recent investigations also show that peripheral blood cells are an effective target for studies of improved glucose uptake and insulin sensitivity. The current research has shown that one of the mechanisms of reduced blood glucose levels achieved by
Chlorella intake is an activation of insulin signaling pathways resulting from changes in gene expression in the peripheral blood cells. This research also suggests, however, that changes in gene expression profile in the peripheral blood can be useful as a surrogate marker when investigating glucose metabolism. This marker is particularly effective in research targeting human beings.

The current research also showed that Chlorella intake in humans is useful in improving fat metabolism. Particularly in the case of the D Group, significant decreases in total blood serum cholesterol, HDL-cholesterol, and LDL-cholesterol were observed at all measurement points after the start of Chlorella intake.

Merchant and Andre (12) used a double blind test to study the effects of Chlorella intake on symptom improvements in a total of 55 patients suffering from fibromyalgia syndrome, hypertension, and ulcerative colitis. They confirmed that Chlorella intake lowers cholesterol in the blood, which is consistent with the outcome of the current research.

Shibata et al. (13) reported that in rats raised on feed containing cholesterol, the administration of Chlorella decreases cholesterol concentrations in the blood and liver, but that there were no changes in neutral fat or phospholipid volumes and that excretion of neutral steroids was increased. They surmised that the cholesterol-lowering effects of Chlorella are brought about by increasing neutral steroid elimination in feces. Sano et al. (14) also reported that in rats with hyperlipemia created through excess administration of cholesterol, the administration of Chlorella increased steroid elimination in the feces. Connor et al. (15) reported that administering high-molecular-weight unsaturated fatty acids to humans increases the neutral steroid content of feces and also reduces blood cholesterol concentrations. Based on the fact that about 74% of the fatty acids contained in Chlorella are unsaturated fatty acids, we can infer that the presence of a physiological mechanism in which Chlorella intake causes the reductions in blood cholesterol as follows: Chlorella intake may increase neutral steroid elimination in the feces, causing a concomitant demand for cholesterol in the liver, which in turn reduces cholesterol concentrations in the blood.

In recent years, the so-called Randle hypothesis (16) was proposed to describe the inhibition of glucose uptake by abnormalities in fat metabolism, stating that increases in fatty acids limit the oxidation and uptake of glucose. This hypothesis has been explained through extensive experimental results. Another approach has also been proposed in skeletal muscle cells whereby the uptake of free fatty acids is stimulated, deactivating the insulin signal transduction systems that transduce signals from insulin receptors to GLUT4. (17) In the current research as well, we can assume that Chlorella intake first improves fat metabolism, resulting in improved glucose uptake. Although cholesterol levels in the blood decrease, triglyceride concentrations do not necessarily drop, so the relationship between the improvement of fat metabolism and the decrease in blood glucose level is unclear at present. It will be necessary to conduct further studies, including in vitro experiments, to elucidate the mechanisms involved.

CONCLUSIONS

In order to clarify the physiological effects of Chlorella intake on subjects with high-risk factors for lifestyle-related diseases, we conducted blood biochemical tests on a high-risk factor group and a healthy subject group and analyzed gene expression profiles in peripheral blood cells before and after Chlorella intake. The results of these tests showed that Chlorella intake brings about improvements in both fat metabolism and glucose metabolism. The expression of genes involved in the insulin signaling pathway was also affected by Chlorella intake, especially those related to glucose uptake in tissue, providing support for the observation that Chlorella lower blood glucose levels. These results indicate that changes in gene expression in the peripheral blood can be useful as a surrogate marker for investigating the mechanisms of modulation of glucose sensitivity in humans. In the clarification of how functional and health foods can contribute to human health, the combination of the nutrigenomics research methods with conventional blood biochemical tests such as those used in this study is being used more widely.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.


REFERENCES


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**TABLE 1. CHARACTERISTICS OF HEALTHY AND HIGH-RISK SUBJECT GROUPS**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Healthy subject group (N Group)</th>
<th>Age (years)</th>
<th>34.3 (+ or -) 3.2</th>
<th>Height (cm)</th>
<th>170.7 (+ or -) 1.6</th>
<th>Body weight (kg)</th>
<th>64.4 (+ or -) 2.2</th>
<th>Body fat percentage</th>
<th>19.8 (+ or -) 1.5</th>
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<tr>
<td>Total cholesterol (mg/dL)</td>
<td>173.8 (+ or -) 12.0</td>
<td>101.2 (+ or -) 11.7</td>
<td>127.4 (+ or -) 11.7</td>
<td>218.4 (+ or -) 38.7</td>
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<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>61.0 (+ or -)</td>
<td>59.5 (+ or -) 12.0</td>
<td>59.3 (+ or -) 21.4</td>
<td>59.5 (+ or -) 12.0</td>
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<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>124.4 (+ or -) 76.6</td>
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<td>124.4 (+ or -) 76.6</td>
<td>124.4 (+ or -) 76.6</td>
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<td>Glucose (mg/dL)</td>
<td>111.4 (+ or -) 30.1</td>
<td>82.4 (+ or -) 7.2</td>
<td>82.4 (+ or -) 7.2</td>
<td>82.4 (+ or -) 7.2</td>
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<td>Body mass index (kg/m²)</td>
<td>24.4 (+ or -) 2.1</td>
<td>21.8 (+ or -) 2.2</td>
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