Attenuating Effect of Chlorella Supplementation on Oxidative Stress and NFκB Activation in Peritoneal Macrophages and Liver of C57BL/6 Mice Fed on an Atherogenic Diet

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This study was designed to investigate whether chlorella supplementation may ameliorate oxidative stress and nuclear factor kappa B (NFκB) activation in peritoneal macrophages and liver of C57BL/6 mice fed on an atherogenic diet. The animals were maintained on an atherogenic diet (control), or an atherogenic diet supplemented with 3% (w/w) chlorella or 5% (w/w) chlorella for 12 wks. The plasma and hepatic lipid levels were not affected by chlorella supplementation. Hepatic thiobarbituric acid-reactive substances and superoxide anion production in peritoneal macrophages were significantly lower in the 5% chlorella group (p<0.05), but the glutathione level was not altered by chlorella supplementation. The hepatic antioxidative enzyme activities of Cu, Zn-superoxide dismutase and catalase were higher in the mice fed on the 5% chlorella diet (p<0.05). The plasma aspartate aminotransferase activity was lower in the mice fed on the chlorella-containing diets (p<0.05), whereas the alanine aminotransferase activity was not affected by chlorella supplementation. The NFκB nuclear binding activities of peritoneal macrophages and liver were significantly lower in the 5% chlorella groups (p<0.05). These results suggest that chlorella supplementation may attenuate oxidative stress by reducing reactive oxygen production and increasing antioxidative processes, thus suppressing inflammatory mediator activation in peritoneal macrophages and liver.

Key words: chlorella; oxidative stress; nuclear factor kappa B (NFκB); C57BL/6 mice; inflammation

Several lines of evidence have been reported for the anti-atherogenic effect of chlorella on rats and rabbits.¹⁻³ Previous studies on the anti-atherogenic effect of chlorella have emphasized the relationship between the serum cholesterol levels and the risk of atherosclerosis. Recent works have revealed that the oxidative stress induced by reactive oxygen species (ROS) and inflammation seem to play an important part in the etiology of this disease.⁴⁻⁶ Numerous lines of evidence suggest that inflammatory macrophages induce oxidative stress by producing such oxidants as the superoxide anion and nitric oxide.⁶⁻⁹ Several animal studies have shown that increased oxidative stress is a mechanism for atherosclerosis.¹⁰⁻¹³ Indeed, Hoffman et al.¹² have found that oxidative stress enhanced nuclear factor kappa B (NFκB) activation and the expression of the vascular cell adhesion molecule-1 and tissue factor in the vasculature of apoE-null mice. These findings seem to indicate that elevated oxidative stress contributes to the development of atherosclerosis through the inflammatory process.

Foodstuffs or food components to attenuate oxidative stress and inflammation have been extensively studied.⁸⁻¹³ The free-radical scavenging, anti-oxidative, anti-inflammatory and anti-tumor activities of chlorella have also been discovered in in vitro studies.¹⁶⁻¹⁹ Chlorophyll and phenolic compounds have been suggested as the active components with antioxidative activity.¹⁹⁻²¹ Previous studies have emphasized that oxidative stress accelerated vascular oxidative injury. If oxidative stress were responsible for the development of vascular inflammation and atherosclerosis, these phenomena might be induced in any tissue that accumulates oxidized lipids or reactive oxygen species.

Macrophages play an essential role in inflammation and premature atherosclerosis by producing pro-inflammatory mediators, trapping oxidized LDL via scavenger receptors, and transforming into foam.

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Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; EMSA, electrophoretic mobility shift assay; ER, endoplasmic reticulum; GSH, glutathione; GSH-px, glutathione peroxidase; NFκB, nuclear factor kappa B; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances
cells. Liver is the organ where the production and degradation of apoB-100-containing lipoproteins takes place. Davis and Hui have proposed that “atherosclerosis is a liver disease of the heart.” Werstuck et al. have also reported that endoplasmic reticulum (ER) stress by oxidative stress resulted in dysregulation of the cholesterol and triglyceride biosynthetic pathways which might be a possible mechanism for atherosclerosis. Furthermore, a few studies have reported that oxidative stress promoted NFκB activation in Kupffer cells and in mice with hepatic steatosis. NFκB is one of the transcription factors that may be controlled by the redox status of the cell and regulates the expression of many target genes involved in immune and inflammatory responses. These previous works led us to investigate whether chlorella supplementation could ameliorate oxidative stress and NFκB activation in peritoneal macrophages and liver of C57BL/6 mice fed on an atherogenic diet for 12 wks.

**Materials and Methods**

**Animals and diets.** This study was conducted in accordance with the Guidelines for Animal Experiments approved by Inje University, Kimhae, Korea. Male C57BL/6 mice (n = 36, 5 wks old) were obtained from Hyochang Science (Taegu, Korea). Two mice each were housed in a cage and maintained in a temperature-controlled (25°C) facility with a 12-h light/dark cycle. They were assigned to control or chlorella diet depending upon the baseline cholesterol level and body weight determined before starting the experiment. To induce oxidative stress, an atherogenic diet was introduced and chlorella was supplementated at the level of 3% or 5% of the total diet. Dried, powdered chlorella (Chlorella ellipsoidea) was obtained from Korea Chlorella Company (Kimhae, Korea). The protein content of the chlorella was 34.8%, lipid was 0.5%, dietary fiber was 10.6%, ash was 21.1%, and carbohydrate was 21.4%. The composition of the experimental diets was manipulated to be isocaloric and is shown in Table 1. The mineral and vitamin mixtures were obtained from ICN Biochemicals (Costa Mesa, CA, U.S.A.). The body weight and food intake were measured every week. The mice were killed by cervical dislocation after blood collection from the retro-orbital sinus, and perfused with 10 ml of a phosphate-buffered saline solution (PBS, pH 7.4) via the left ventricle. The liver was frozen in liquid nitrogen and stored in −70°C until needed for analysis.

**Peritoneal macrophage production and culture.** Intraperitoneal injection of thioglycollate (1 ml of a 4% solution) was used to recruit peritoneal phagocytes. These cells were harvested 3 d later by lavage with PBS. The cells were pelleted by centrifugation at 500 × g for 5 min, washed with PBS, and cultured in Dulbecco’s modified Eagle’s medium supplemented with 2 mM glutamine, 10% (v/v) fetal bovine serum, 100 U/ml of penicillin and 0.1 mg/ml of streptomycin. Macrophages were purified by their adherence to tissue culture plates for 4 h, these being used for measurements of the superoxide anion level and NFκB activity.

**Lipid assay.** The total cholesterol, triglyceride, and HDL-cholesterol concentrations in the plasma were determined by colorimetric kits (Sigma, St. Louis, MO, U.S.A.). Hepatic lipids were extracted by the method of Folch et al. The dried lipid residue was dissolved in 1 ml of chloroform with Triton X-100, evaporated under N2, and then dissolved in 1 ml of distilled water. The hepatic cholesterol and triglyceride levels were analyzed by the enzymatic kits used for the plasma analysis.

**Lipid peroxidation.** Lipid peroxidation in the plasma and liver was quantified by measuring the levels of thiobarbituric acid-reactive substances (TBARS) as described by Fraga et al. The TBARS values are expressed as nmol of malondialdehyde equivalents per g of tissue, using a standard curve for 1,1,3,3-tetraethoxypropane.

**Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) assays.** The activities of ALT and AST, which are markers of the hepatic function, were measured by diagnostic kits Sigma (St. Louis, MO, U.S.A.).

**Superoxide anion production.** Aliquots containing 5 × 10⁶ peritoneal macrophages were mixed with ferricytochrome C (1 mg/ml) and stimulated by the addition of-phorbol myristate acetate (400 nmol/l). Superoxide production was measured at 37°C as the change in absorbance at 550 nm by a Powerwave HT microplate reader (Bio-Tek, VT, U.S.A.). The absorbance was read at 1-min intervals up to 10 min.

<table>
<thead>
<tr>
<th>Table 1. Composition of the Experimental Diets (%)</th>
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<tbody>
<tr>
<td>Control diet</td>
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<tr>
<td>---------------</td>
</tr>
<tr>
<td>Casein</td>
</tr>
<tr>
<td>Sucrose</td>
</tr>
<tr>
<td>Corn starch</td>
</tr>
<tr>
<td>Cocoa butter</td>
</tr>
<tr>
<td>Corn oil</td>
</tr>
<tr>
<td>Cholesterol</td>
</tr>
<tr>
<td>DL-methionine</td>
</tr>
<tr>
<td>Vitamins</td>
</tr>
<tr>
<td>Minerals</td>
</tr>
<tr>
<td>Cellulose</td>
</tr>
<tr>
<td>Sodium cholate</td>
</tr>
<tr>
<td>Choline bitartrate</td>
</tr>
<tr>
<td>Chlorella powder</td>
</tr>
</tbody>
</table>

Daviso and Hui have proposed that atherosclerosis is a liver disease of the heart. Werstuck et al. have also reported that endoplasmic reticulum (ER) stress by oxidative stress resulted in dysregulation of the cholesterol and triglyceride biosynthetic pathways which might be a possible mechanism for atherosclerosis.
Glutathione (GSH) concentration. Total GSH was measured by the enzymatic recycling procedure of Tietze, in which GSH is sequentially oxidized by 5,5'-dithiobis (2-nitrobenzoic acid) and reduced by NADPH in the presence of glutathione reductase. The rate of 2-nitro-5-thio-benzoic acid formation was monitored spectrophotometrically at 412 nm. The GSH content was determined by comparing the measured rate to a standard curve generated with a known amount of GSH.

Antioxidative enzyme activities. The liver samples were homogenized in 20 parts (w/v) of a 50 mM phosphate buffer (pH 7.4), using a tissue homogenizer with a Teflon pestle at 4°C. The homogenate was centrifuged at 600 × g for 10 min to discard the cell debris, and the resulting supernatant was further centrifuged at 10,000 × g for 20 min to separate the mitochondrial pellet and cytosolic fraction. The protein concentration of each fraction was measured by the Bradford assay. With bovine serum albumin used as the standard. The Cu,Zn-superoxide dismutase (SOD) and Mn-SOD activities were measured by the Marklund and Marklund method. The catalase activity was measured by the Aebi method, and the activities of glutathione peroxidase (GSH-px) and glutathione reductase were respectively measured by the methods of Lawrence and Burk and Goldberg and Spoone.

Electrophoretic mobility shift assay (EMSA). Nuclear protein was extracted by slight modification the method of Dignam et al. Peritoneal macrophages and a portion of the liver were lysed or homogenized with a buffer containing 0.6% igepal, 0.15 mM NaCl, 10 mM Tris at pH 7.9, 1 mM EDTA and a 0.1% protein inhibitor cocktail, vortexed, kept on ice for 5 min, and centrifuged at 500 × g for 5 min at 4°C. The pelleted nuclei were resuspended in 60 μl of an extraction buffer [10 mM Hepes at pH 7.9, 0.1 mM EDTA, 1.5 mM MgCl2, 420 mM NaCl, 25% glycerol, 1 mM dithiothreitol, and a 0.3% protein inhibitor cocktail]. After gently mixing and keeping on ice for 20 min, the samples were centrifuged at 500 × g for 5 min at 4°C. The supernatant fraction was transferred to new tubes and stored at −70°C. The protein concentration was determined by the Bradford assay.

The NFκB-specific oligonucleotide was end-labeled with [32P]-ATP by using T4 polynucleotide kinase (Promega, Madison, WI, U.S.A.) and purified in a microspin G-25 column (Amersham, Piscataway, NJ, U.S.A), before EMSA was performed according to the instruction manual of Promega. Five to ten μg of nuclear protein and 2–4 μl of a binding buffer were mixed, and the mixture incubated for 10 min on ice. A 1–μl amount of 32P-labeled NFκB and 2 μl of the loading buffer were added to the nuclear protein mixture, and the resulting mixture was incubated for 30 min at room temperature. Competition assays used a 100-fold excess of unlabeled NFκB that was added 10 min before adding the radiolabeled probe. The nuclear proteins were incubated with 2 g of anti-p50 and anti-p65 at room temperature for the supershift assay. The DNA-protein complexes were separated from the unbound DNA probe by electrophoresis through 4% polyacrylamide gel by using 0.5× Tris-borate-EDTA (pH 8.0) as the running buffer. The gels were dried and exposed on an X-ray film for 2 h at room temperature, and the resulting bands were quantitated by a phospho imager (Packard, U.S.A.).

Statistical analysis. Data are expressed as the mean ± SEM, and statistical analyses were performed by the SPSS program. One-way ANOVA and Duncan’s multiple-range test were used to examine the difference between groups, statistical significance being considered at P < 0.05.

Results

Weight gain, food intake, and food efficiency, and liver weight

The initial body weights of the mice were similar between those fed on the atherogenic diets supplemented with 3% or 5% chlorella (chlorella groups) and the chlorella-free diet (control group). The weight gain, food intake, and food efficiency during this study were no different among the groups (Table 2), suggesting that chlorella supplementation did not affect the appetite or growth of the C57BL/6 mice. The liver weight of the mice fed on the chlorella diets (3%, 1.55 g; 5%, 1.68 g) was not significantly different from those fed on the control diet (1.65 g).
Table 3. Effect of Chlorella Supplementation on the Plasma and Hepatic Lipid Levels in C57BL/6 Mice Fed on the Atherogenic Diet\(^1\)

<table>
<thead>
<tr>
<th></th>
<th>Control diet</th>
<th>3% chlorella diet</th>
<th>5% chlorella diet</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>193 ± 11(^{NS})(^2)</td>
<td>164 ± 12</td>
<td>170 ± 8</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl)</td>
<td>43.7 ± 2.6(^{NS})</td>
<td>42.1 ± 3.1</td>
<td>36.6 ± 3.4</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>28.1 ± 2.7(^{NS})</td>
<td>30.6 ± 1.6</td>
<td>27.0 ± 1.3</td>
</tr>
<tr>
<td><strong>Liver</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol (mg/g)</td>
<td>6.09 ± 0.61(^{NS})</td>
<td>5.79 ± 0.52</td>
<td>6.27 ± 0.46</td>
</tr>
<tr>
<td>Triglyceride (mg/g)</td>
<td>2.46 ± 0.28(^{NS})</td>
<td>3.10 ± 0.15</td>
<td>2.53 ± 0.24</td>
</tr>
</tbody>
</table>

\(^1\) Each value is the mean ± SEM of 12 mice.  
\(^2\) Not significant.

Table 4. Effect of Chlorella Supplementation on the TBARS and GSH Concentrations and AST and ALT Activities in the Plasma and Liver of C57BL/6 Mice Fed on the Atherogenic Diet\(^3\)

<table>
<thead>
<tr>
<th></th>
<th>Control diet</th>
<th>3% chlorella diet</th>
<th>5% chlorella diet</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBARS (nmol MDA/ml)</td>
<td>13.1 ± 0.5(^{NS})(^2)</td>
<td>12.2 ± 0.9</td>
<td>10.9 ± 1.4</td>
</tr>
<tr>
<td><strong>Hepatic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBARS (nmol MDA/g)</td>
<td>18.1 ± 1.2(^{NS})(^3)</td>
<td>19.1 ± 0.8(^a)</td>
<td>13.9 ± 0.9(^p)</td>
</tr>
<tr>
<td>GSH ((\mu)mol/g)</td>
<td>4.39 ± 0.15(^{NS})</td>
<td>4.61 ± 0.27</td>
<td>4.41 ± 0.32</td>
</tr>
<tr>
<td><strong>AST (unit/l)</strong></td>
<td>104.8 ± 6.1(^c)</td>
<td>77.3 ± 6.4(^d)</td>
<td>72.8 ± 7.3(^p)</td>
</tr>
<tr>
<td><strong>ALT (unit/l)</strong></td>
<td>56.6 ± 8.3(^{NS})</td>
<td>52.8 ± 6.5</td>
<td>44.6 ± 5.2</td>
</tr>
</tbody>
</table>

\(^1\) Each value is the mean ± SEM of 12 mice.  
\(^2\) Not significant.  
\(^3\) Values in a row with same letters are not significantly different at \(P < 0.05\).

Lipid levels in the plasma and liver

The lipid levels in the plasma and liver of the C57BL/6 mice after 12 wks of diet feeding are shown in Table 3. After 12 wks of feeding, the plasma total cholesterol level had increased almost 2 fold in all groups. However, the final plasma total cholesterol, triglyceride, and HDL-cholesterol levels were no very different among the groups, although the cholesterol level of the 5% chlorella group was slightly lower than that of the control group, showing significance at \(p < 0.1\). Furthermore, chlorella supplementation did not suppress the increase in total cholesterol and triglyceride concentrations in the liver of the C57BL/6 mice fed on the atherogenic diet. These data indicate that chlorella supplementation up to 5% did not affect the lipid levels in the plasma and liver of C57 mice fed on the atherogenic diet.

Oxidative stress status and liver function

The level of oxidative stress was determined by the status of TBARS, GSH, and the superoxide anion in the plasma, liver, and peritoneal macrophages. The levels of plasma TBARS and hepatic GSH were not significantly different among the groups. However, the 5% chlorella supplementation significantly suppressed the TBARS level in the liver homogenate \((p < 0.05)\) (Table 4). Macrophages isolated from the control group demonstrated a burst of superoxide production. In contrast, peritoneal macrophages isolated from the mice fed on the chlorella diets showed suppressed superoxide anion production in a dose-dependent manner. The degradation of endogenous and/or produced superoxide anion in the late stage of incubation was monitored in macrophages of the chlorella groups (Fig. 1). The plasma AST activity, a biomarker of the liver function, was significantly lower in the mice fed with the chlorella-supplemented diets \((p < 0.05)\), whereas the ALT activity was not altered by chlorella supplementation (Table 4).

Antioxidative enzyme activities

The activities of the hepatic antioxidative enzymes are shown in Table 5. The activities of Cu,Zn-SOD and catalase were significantly higher in the 5%
Table 5. Effect of Chlorella Supplementation on the Hepatic Antioxidative Enzyme Activities in C57BL/6 Mice Fed on the Atherogenic Diet1)

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>Control diet (unit/mg of protein)</th>
<th>3% chlorella diet (unit/mg of protein)</th>
<th>5% chlorella diet (unit/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu,Zn-SOD</td>
<td>8.5 ± 1.5a</td>
<td>10.8 ± 0.9a</td>
<td>24.1 ± 1.9b</td>
</tr>
<tr>
<td>Mn-SOD</td>
<td>1.7 ± 0.1a</td>
<td>1.5 ± 0.1a</td>
<td>1.8 ± 0.1b</td>
</tr>
<tr>
<td>Catalase</td>
<td>2.0 ± 0.2a</td>
<td>2.9 ± 0.2a</td>
<td>2.6 ± 0.1b</td>
</tr>
<tr>
<td>Glutathione peroxidase</td>
<td>299 ± 10a</td>
<td>289 ± 19a</td>
<td>246 ± 11b</td>
</tr>
<tr>
<td>Glutathione reductase</td>
<td>71.1 ± 0.04a</td>
<td>71.1 ± 0.03</td>
<td>71.2 ± 0.04</td>
</tr>
</tbody>
</table>

1) Each value is the mean ± SEM of 12 mice.
2) Superoxide dismutase.
3) Values in a row with the same letters are not significantly different at \( P < 0.05 \).
4) Not significant.

Fig. 2. NFκB Binding Activity of Macrophage Nuclear Extracts.

(A) Supershift assay of NFκB. Lane 1, nuclear extracts from macrophages; lane 2, nuclear extracts from macrophages incubated with 100-fold unlabeled NFκB nontspecific oligonucleotide; lanes 3–5, nuclear extracts from macrophages with 2 mg of normal rabbit serum, anti-p65, and anti-p50, respectively. Circles indicate the bands shifted by the antisera. The arrow indicates p50/p65 heterodimeric NFκB. (B) NFκB binding activity of the nuclear extracts of peritoneal macrophages harvested from mice fed on the control, 3% chlorella, and 5% chlorella diets for 12 wk. Lanes 1–3: control, 3% chlorella, and 5% chlorella diet groups, respectively (n = 6). (C) Each value is expressed as the relative intensity of radioactivity. Bars with same appended letters are not significantly different at \( P < 0.05 \).

Nuclear-binding activity of NFκB

Figure 2 shows the NFκB nuclear-binding activity of peritoneal macrophages harvested from the mice fed on the experimental diets. The nuclear-binding activity of NFκB in the peritoneal nuclear extract was significantly lower in the 5% chlorella group (p < 0.05) (Figs. 2B and 2C). The specificity of the binding was assessed by competition with a 100-fold molar excess of the unlabeled NFκB oligonucleotide. The antibody specific to the p50 and p65 subunits of NFκB super-shifted the upper band, but not the lower band (Fig. 2A). The lower band was not identified, but could have represented inactivated NFκB bound with inhibitor protein IkB or the monomeric p52 component of NFκB.35) Figure 3 shows the hepatic nuclear-binding activity of NFκB. A positive control (HeLa extract, Promega) indicates the position of the NFκB-oligonucleotide complex (Fig. 3A). The NFκB binding activity was significantly lower (p < 0.05) in the hepatic nuclear extracts isolated from mice that had been fed on the 5% chlorella diet (Figs. 3B and 3C).

Discussion

We examined in this study the protective effect of chlorella supplementation on the atherogenic diet-induced oxidative stress and NFκB activation in peritoneal macrophages and the liver of C57BL/6 mice. The results of our study demonstrate that 5% chlorella supplementation to C57BL/6 mice significantly attenuated the NFκB activation and superoxide anion production in thioglycollate-elicited peritoneal macrophages. Although there is no consensus as to the pathogenetic significance of NFκB in macrophages, there is some evidence that superoxide anion production was increased in macrophages of atherosclerotic lesions upon NFκB activation.6,9) Accordingly, the suppression of superoxide anion...
production in macrophages isolated from the 5% chlorella group might have been due to attenuated NFκB activation in the macrophages. This evidence strongly indicates that chlorella supplementation ameliorated the inflammatory process in peritoneal macrophages.

It has been proposed that NFκB activation in macrophages/monocytes was stimulated by oxidative stress. Furthermore, it has been reported that carnosol, an antioxidant in rosemary, suppressed NFκB activation and that supplementation with epigallocatechin-3-gallat and vitamin E suppressed superoxide anion generation in macrophages. This evidence suggests that the suppression of superoxide anion production and NFκB activation in peritoneal macrophages of mice fed on a chlorella diet might be attributable in part to the antioxidative properties of chlorella. Several lines of evidence have suggested the free radical-scavenging activities of chlorella, which may have been due to the presence of chlorophyll, phenol compounds of the methanolic extract, or hydrophilic components present in the aqueous extract of chlorella. It has been reported that chlorophyllin, soluble form of chlorophyll, had potent antioxidative ability involving scavenging of various physiologically important ROS. It is quite certain from this evidence that several compounds in chlorella, whether lipophilic or hydrophilic, may combat oxidative stress.

It is well known that an atherogenic diet promotes hyperlipidemia, lipid peroxidation, and NFκB activation that may contribute to the initiation of the atherosclerotic process. We observed lower NFκB activation in the liver of mice fed on the atherogenic diet supplemented with 5% chlorella. Since NFκB is a redox-regulated transcription factor, the attenuated NFκB activity might have been due to the lower oxidative stress in the liver of the 5% chlorella group. Indeed, the hepatic TBARS level was lower in the mice fed on the 5% chlorella supplemented diet, which means less lipid peroxidation. Furthermore, the higher activity of such hepatic antioxidative enzymes as Cu,Zn-SOD and catalase was observed in the mice fed on the 5% chlorella diet than in those fed on the control diet. Such intracellular antioxidative enzyme as SOD and catalase prevent cells from free radical-mediated disturbance by scavenging ROS and products of lipid peroxidation. These results suggest that chlorella supplementation attenuated hepatic oxidative stress from the atherogenic diet through the direct antioxidative action of chlorella and/or by enhanced antioxidative enzyme activity to ameliorate the superoxide anion and H2O2 toxicity, which subsequently resulted in suppressed NFκB activation. It has been reported that elevated oxidative stress depleted the GSH pool, the front line in antioxidative processes. In this study, the hepatic GSH concentration was not altered by chlorella supplementation, which indicates that chlorella supplementation attenuated the oxidative stress without altering the GSH status. A similar observation has been made by Balkan et al., who observed that the hepatic GSH level was unaltered, but that the TBARS concentration was decreased by taurine supplementation to rabbits fed on a high-cholesterol diet.

We found that chlorella supplementation did not affect the lipid levels in the plasma and liver of C57BL/6 mice fed on an atherogenic diet. However, the hypocholesterolemic effect of chlorella or chlorella extracts have been reported on rabbits and rats. Shibata et al. have suggested that the hypocholesterolemic effect of the indigestible fraction of chlorella was due to the enhanced fecal neutral steroid excretion which potentiated the role of dietary fiber from chlorella in reducing the plasma cholesterol level. In our study, the content of dietary fiber was corrected to be equal among the diet any treatments to prevent any calorie-dilution effect. Accordingly, the discrepancies between our result and others might have arisen from differences in the animal strain, diet composition, feeding period, and concentration of chlorella fed.

**Fig. 3.** NFκB Binding Activity of Hepatic Nuclear Extracts. (A) Supershift assay of NFκB. Lane 1, HeLa-positive extract; lanes 2–3, HeLa nuclear extract incubated with 50- and 100-fold unlabeled NFκB nonspecific oligonucleotide; lane 4, HeLa nuclear extract incubated with 2 mg of the anti-p65 serum. The arrow indicates p50 NFκB dimeric NFκB. (B) NFκB binding activity of the nuclear extract of liver from mice fed on the chlorella and control diets for 12 wk (n = 6). Lanes 1 and 2, control; lanes 3 and 4, 3% chlorella; and lanes 5 and 6, 5% chlorella. (C) Each value is expressed as relative intensity of radioactivity. Bars with same appended letters are not significantly different at P < 0.05.
It is postulated that damage to the liver by oxidative stress might be potentiated by NFκB activation. Indeed, NFκB activation and a concomitant rise in oxidative stress in the cholestatic liver of bile duct-ligated mice\(^{25,40}\) and Kupffer cells\(^{50}\) have been reported. NFκB activation in damaged liver has been explained as a pathological adaptation to help survival and the continued function of hepatocytes.\(^{25}\) Our biochemical analysis of AST activity showed that chlorella supplementation reduced the damaged liver function due to oxidative stress, this result being supported by Wang et al.\(^{40}\) who have reported that chlorella-fed rats were less susceptible and recovered more quickly from toxic injury than the controls by an ethionine treatment. Therefore, the suppressive effect of chlorella supplementation on NFκB activation and AST activity in the liver might provide a potential new mechanism to explain the beneficial effects of chlorella intake in preventing liver diseases.

In summary, our results indicate that chlorella supplementation may improve the disturbance of ROS generation, lipid peroxidation, and inflammatory mediator activation as possible mechanisms for its anti-atherogenic influence. Thus, chlorella supplementation as an antioxidative therapy may be beneficial for preventing atherosclerosis due to enhanced intracellular oxidative stress and inflammation. Further studies to identify the effective components of chlorella have to be made.

Acknowledgments

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