糖尿病合併症における酸化ストレスの関与と食品因子による予防のメカニズムの解析

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研究概要

糖尿病合併症の発症における酸化ストレスの関与と食品因子による予防のメカニズムの解析について分子レベルからの研究を進め、次のような研究実績を得ることができた。つい最近の報道では、日本人の糖尿病の患者は予備軍も含めると１３００万人以上にものぼるというショッキングなニュースが全国を駆け巡った。一般に糖尿病はインスリン依存型糖尿病（ＩＤＤＭ）とインスリン非依存型糖尿病（ＮＩＤＤＭ）に大別することができる。ＩＤＤＭは短期間に内因性インスリン分泌の急激な欠乏により生命維持が困難となってしまう病態であり、ＮＩＤＤＭは内因性インスリン分泌能がすくなくとも保持されている点で異なっている。現在、世界的に糖尿病、特に、ＮＩＤＤＭが急増しており、工業化・近代化に伴う生活習慣、特に食習慣の変容、なかでも過食と運動不足、その結果の肥満が問題となっている。糖尿病が重大な病気として考えられている背景には、合併症の存在が問題とされるが、一般に、糖尿病の合併症としては、急性合併症と慢性合併症に大別され、代表的な急性合併症である糖尿病性昏睡や急性感染症などはインスリン療法の進歩により著しく改善されてきたが、高血糖が持続するために生じた様々な代謝異常によって引き起こされる慢性合併症は糖尿病合併症の主流となってきており、網膜症や腎症などの細小血管症や動脈硬化にもとずく大血管症などの病態がますます重大な状況となってきている。

このような糖尿病の発症とフリーラジカルの関連性は、最近、特に注目を集めつつある分野であり、なかでも糖尿病の進展に活性酸素が大きく関与していると考えられている。最近、われわれの研究室では動脈硬化症の発症の原因における酸化ストレスの役割について免疫化学的なアプローチを中心に解明を進めつつある。本研究では、このような酸化ストレスが本当に糖尿病合併症の原因となりうるのか、また、坑酸化食品因子による糖尿病合併症予防は期待できるのか、検討を行うことを目的とする。

１．　糖尿病と酸化ストレス

糖尿病と酸化ストレスの関連性には最近多くの注目が集められてきている。なぜ、糖尿病合併症の発症と共に酸化ストレスが昂進するのかと言えば、高血糖状態が続くと、生体構成タンパク質の糖化反応やポリオール代謝とレドックス、プロスタグランジン代謝などの経路と共に、グルコースの自動酸化などの経路により活性酸素が生成し、動脈硬化をはじめ腎障害、糖尿病性白内障などの原因となると考えられている。今までの多くの研究により、糖尿病の発症におけるアミノカルボニル反応（メイラード反応）と呼ばれるタンパク質の非酵素的な糖付加反応の役割の重要性が明らかとなってきた。その結果、グルコースに代表される還元糖がタンパク質のアミノ基を攻撃し、シッフ塩基やアマドリ転移生成物といったメイラード反応前期生成物を経て、後期反応の進展の結果、ピラリン(pyrraline)やペントシジン(Pentosidine)、クロスリン(Crossline)などの AGE(Advanced Glycation End Products)とよばれるメイラード反応終期生成物を生成することが知られている。著者

らも、糖尿病患者や自然発症糖尿病ラットの血液中にアルギニンとシステインが関与した新しいAGE、MRXの構造を明らかにすることに成功している。このメイラード反応の前期反応生成物であるアマドリ転移生成物は、試験管内でも生体内でも活性酸素を生成し、生体内タンパク質や脂質、DNAに傷害を与えることが明らかにされてきている。 著者らは、糖尿病における酸化ストレスの傷害の役割を検討する目的で、ストレプトゾトシン(STZ)誘発糖尿病ラットを用い、フリーラジカル傷害バイオマーカーとして細胞膜脂質の過酸化物やDNAの酸化傷害物として近年注目を集めている8-ヒドロキシデオキシグアノシン(8-OH-dG)の検出を試みた。8-OH-dGは、がんを始め成人病のマーカーとしての遺伝子レベルにおける酸化的傷害の評価法として、最近、特に注目を集めてきている。今まで、放射線や紫外線をはじめさまざまな化学物質も生体内での酸化ストレスの原因であることが知られてきており、なかでも、DNAの核酸塩基の酸化修飾に多くの注目が集められてきている。特に、生体内脂質過酸化反応の結果生じた活性酸素によるDNA中の核酸塩基、デオキシグアノシンに対する攻撃の結果、8-ヒドロキシデオキシグアノシン（8-OHdG）が生成するというわけである。もちろん、8-OHdGは過剰な酸化ストレスを受けた皮膚細胞や腎細胞中でも蓄積することも明らかにされてきたが、このDNA中に生じた8-OHdGは、通常は修復酵素により切り取られ、血液をへて最終的に尿中に排出されることが明らかになってきた。従って、血液や尿中の8-OHdG量を測定することは成人病予防の重要なバイオマーカーとなりうるわけで、日本老化制御研究所と共同でこの抗体を利用したELISA法による微量分析キットの作製にも成功することができた。この8-OH-dGは、紫外線照射による皮膚がんの発症や鉄キレート化合物の投与による腎臓がん発症の際に増加することを明らかにしてきており、また、ELISA法による微量定量法の確立にも成功している。このような免疫化学的なアプローチによる酸化ストレスの評価については、まだ、始まったばかりであるが、今後の進展に興味がもたれる。

しかしながら、最終的には遺伝子レベルに至る酸化傷害が問題となるが、まず最初の酸化ストレスのターゲットとなるのは、細胞の生体膜を構成する不飽和脂肪酸の過酸化物である。一般的には、不飽和脂肪酸の二重結合の間の活性メチレンが活性酸素による水素引き抜き反応を受け、脂質ラジカルを生成し、その後の分子状の酸素の付加によりヒドロペルオキシドが初期反応生成物として生じる。必須脂肪酸であるリノール酸の過酸化物、ヒドロペルオキシドはさらに生体内の金属やヘム蛋白などの存在で酸化分解を引き起こし、マロンアルデヒド(MDA)や4-ヒドロキシ-2-ノネナール (HNE)、アクロレイン などのアルデヒドをはじめとする多種多様なアルデヒド類が生成される。いままでにさまざまな脂質過酸化測定法が開発されてきているが、最近、われわれの研究室で特に注目してきたのは、酸化ストレスの高感度で簡便な評価法の開発であり、なかでも、重点的に研究を進めているのが脂質酸化分解物に特異的な抗体を利用した免疫化学的な微量定量法の確立である。特に著者らが注目したのは、攻撃の対象である脂質、タンパク質、核酸の酸化修飾物をエピトープとする免疫化学的な検出法の確立である。われわれの研究室では、最近、脂質過

酸化初期反応生成物である 13-リノール酸ヒドロキペルオキシド(13-HPODE)をエピトープとするポリクローナル抗体を得ることに成功している。今まで、生体内脂質過酸化反応終期生成物としてよく知られている MDA や HNE による生体傷害については多くの研究が行われてきたが、脂質過酸化初期反応生成物であるヒドロペルオキシドの持つ酸化傷害に及ぼす影響についてはほとんど報告がなされていなかった。この抗体の特異性を検討したところ、エピトープの構造の一つの化学構造を明らかにすることができ、しかも、これらの抗体を用いた化学的な解析の結果、LDL 酸化によりヒドロペルオキシドが生成するという興味ある結果をえることができた。また、最近では、アラキドン酸のヒドロペルオキシド（15-HPETE）の抗体の作製にも成功しており、現在、動脈硬化発症における脂質ヒドロペルオキシドの関与の可能性についての検討を進めている。さらに、われわれの研究室では、n-６系列脂肪酸の酸化終期生成物の MDA と HNE を化学的に合成してタンパク質と反応させることで縮合物を合成し、ポリクローナル抗体を得ることに成功している。また、最近では生体内脂質過酸化反応終期生成物である HNE の場合はモノクローナル抗体を得ることにも成功しているが、このような抗体を用いる利点は、簡便かつ微量で定量できる ELISA 法を構築することができることであり、試験管レベルから培養細胞、個体レベルからヒトを対象とした臨床レベルでも適用することができる点である。これらの抗体はいずれもエピトープが分子レベルで明確にされており、免疫染色法へ応用することにより病態解析の有力な手段となりうる。

本研究の遂行の過程で、われわれはリノール酸ヒドロペルオキシドがたんぱく質中のリジンを修飾したエピトープ構造、ヘキサノニルリジンの構造を化学的に明らかにすることに成功し、モノクローナル抗体の作製に成功した。この内容は、発表論文（２０）としてリストしてあるので、ここでは、そのオリジナルの論文で詳細を紹介したい。また、活性酸素の直接的な酸化修飾の系として、マクロファージや好中球にも注目した。これらは、生体防御機構として病原菌やウイルスを活性酸素により攻撃するが、何らかの理由で過剰発現することが知られている。糖尿病の合併症である白内障や腎不全などの進展にも大きく関係しているものと考えられている。われわれは、特に、好中球の過剰発現で起こるたんぱく質中のチロシン残基の酸化修飾物、ジチロシンについてもモノクローナル抗体の作製に成功し、論文（２１）として発表している。この詳細も、オリジナル論文として紹介したい。

ヘキサノニルリジン構造を認識するモノクローナル抗体の作製と応用

ABSTRACT

The monoclonal antibody to N^{ε}-(hexanonyl)lysine (HEL), a novel adduct formed by the reaction of linoleic acid hydroperoxide and lysine, has been prepared and characterized. The obtained antibody specifically recognized the HEL moiety. Using the monoclonal antibody, we evaluated the protective effects of feeding eriocitrin, which is one of flavonoids in lemon fruit, on oxidative modification induced by exercise in rats. The supplementation of eriocitrin significantly suppressed the increase in HEL in the skeletal muscle by exercise. The result suggests that the determination of HEL may be a good method for evaluation of the protective effect of beneficial food factors against oxidative stress.

Key Words: monoclonal antibody, lipid hydroperoxide, exercise, oxidative stress, flavonoid, biomarker, adduct.

INTRODUCTION

Many diseases are accompanied by lipid peroxidation of lesions. Aldehydes are one of the advanced lipid peroxidation products and can be covalently bound to proteins (1). It has been considered that the covalent protein modification can happen during an even earlier stage of lipid peroxidation (2). Recently, the preparation of polyclonal antibodies to lipid hydroperoxide modified-proteins has been reported (3–5). We have also identified N^{ε}-(hexanonyl)lysine, named HEL, from the reaction between the lysine moiety and 13-hydroperoxyoctadecadienoic acid (13-HPODE) (6).

In recent years, exercise has expanded the scope for prevention and/or management of chronic diseases and maintenance of optimal health. Exercise is recommended in the prevention/management of non insulin-dependent diabetes, hypertension, and coronary heart disease (7, 8). On the other hand, strenuous aerobic exercise is associated with oxidative stress and generates oxygen free radicals (9).

Epidemiological studies showed that the dietary intake of flavonoids was inversely associated with mortality from coronary heart disease (10) and the incidence of stroke (11). These correlations may implicate in part the inhibition of low density lipoprotein (LDL) oxidation and platelet aggregation activity due to the ability of flavonoids to scavenge free radicals or to chelate metal ions (12, 13). We have been involved in the isolation of antioxidative flavonoids from lemon fruit and have identified eriocitrin (eriodictyol 7-*O*-β-rutinoside) (14), which has a stronger antioxidative activity than the other citrus flavonoid compounds (15). Our previous study demonstrated that dietary eriocitrin suppressed oxidative stress in diabetic rats induced by streptozotocin (16).

In this paper, the preparation and characterization of monoclonal antibodies to HEL is reported and the HEL content in the skeletal muscle of the exercise-trained rats with or without feeding of eriocitrin was measured using the antibodies.

MATERIALS AND METHODS

Materials. Keyhole limpet hemocyanin (KLH), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and *N*-hydroxysulfosuccinimide (sulfo-NHS) were obtained from Pierce Chemical Co., Rockford, IL. Peptidase (from porcine intestinal mucosa), protease (from *Streptomyces griseus*), *tert*-butoxycarbonyl-L-lysine (Boc-Lys), and lipid-free bovine serum albumin (BSA) were purchased from Sigma Chemical Co., St. Louis, MO. Benzoyl-glycyl-L-lysine (Bz-Gly-Lys) was purchased from Peptide, Inc., Osaka. Anti-HEL polyclonal antibodies were prepared as described previously (6). Eriocitrin was prepared from lemon peel extracts (14); the purity of eriocitrin was more than 99.0% as analyzed by HPLC.

Immunization and antibody preparation. Hexanonyl KLH and its analog, hexanonyl BSA, were prepared from hexanoic acid and proteins with EDC and sulfo-NHS as described (6). Balb/c mice were primed intraperitoneally with the modified KLH emulsified in a Freund's complete adjuvant. Mice were boosted twice at a 2-week interval. Three days after an intraperitoneal final injection of the antigen without adjuvant, the animals were sacrificed, and the P8U1 myeloma cell line and the spleens of immunized mice were fused using polyethylene glycol. The production and selection of hybridomas were carried out by the method of Kohler and Milstein (17). The supernatants of the culture were used for enzyme-linked immunosorbent assay (ELISA). The positive clones were selected using hexanonyl BSA as the antigen for ELISA. The hybridomas obtained were injected intraperitoneally into Balb/c mice, which had been injected with pristane (2, 6, 10, 14-tetramethylpentadecane) one week before. About 7–10 days later, the ascites were collected and then partially purified by ammonium sulfate fractionation.

Estimation of N^{ε}-(hexanonyl)lysine in hexanonylated protein. The hexanonyl BSA (0.5 mg/ml) was enzymatically hydrolyzed by peptidase (0.01 mg/ml) in 0.1 M phosphate buffer (pH 7.4) containing 0.025% NaN_3 at 37°C overnight (18). Further, additional peptidase (0.01 mg/ml) was used and reacted for 6 h at 37°C. Protease (0.01 mg/ml) was then added and the mixture further incubated at 37°C overnight. The sample was centrifuged at 8,000 rpm for 10 min at 4°C, and the supernatant was collected and then filtered with a cellulose acetate membrane filter (0.45 μm pore). The filtrates were directly applied to an amino acid analyzer as described below. For liquid chromatography-mass spectrometry (LC-MS) analysis, the hydrolysates were cleaned by

an ion-exchange column (Dowex 50W x 8, 100–200 mesh, H^+ form) and acetylated as follows. The hydrolysates were dissolved in 0.1 N HCl and applied to the column (1 ml gel volume). The column was washed with water (more than 5 ml) until the eluent reached a neutral pH. Two milliliters of 7 M NH_4OH were applied and the eluent was collected, evaporated, and freeze-dried. The hydrolysates/amino acids were dissolved in 100 µl of half-saturated sodium acetate, and dehydrated acetate (10 µl) was added three times. The acetylated hydrolysates were used for LC-MS as described.

Preparation of chemically-modified proteins and N^{ε}-(hexanonyl)lysine derivatives.

Aldehyde-modified BSAs were prepared using malondialdehyde, 1-hexanal, and 2-hexenal, and synthetic 4-hydroxy-2-nonenal as described previously (19). Peroxidized lipid-modified BSA, lipid hydroperoxide-modified BSA, and the modified BSA having various alkyl chain lengths in the amide-type adduct were prepared as described (6). Free HEL was prepared from Bz-N^{ε}-(hexanonyl)lysine as follows. Boc-lysine was reacted with hexanoic acid with EDC and NHS as described (6). The obtained N^{ε}-(hexanonyl)lysine derivative was completely dried and, to remove the N^{α}-Boc moiety, trifluoroacetic acid (2 ml) was added and the mixture was further incubated at room temperature for 1 h. After drying the solution, the presence of free HEL was confirmed by both LC-MS and amino acid analysis as described in the section below.

Enzyme-linked immunosorbent assay (ELISA). The competitive indirect ELISA was done as described (6) with some modification, Briefly, 50 µl of hexanonylated BSA (5 µg/ml phosphate-buffered saline (PBS)) were pipetted into the wells and kept at 4°C overnight. At the same time, 50 µl of antibody (0.5 µg/ml) and 50 µl of sample (competitor) were mixed in a tube and reacted at 4°C overnight. The plate was washed, blocked with 4% Block Ace (Dainihon Seiyaku Co., Osaka), and 90 µl of the reacted solution were then pipetted into a well and further incubated. The binding of the residual antibody on the coated modified BSA was estimated using peroxidase-labeled anti-mouse IgG antibody with *o*-phenylenediamine and hydrogen peroxide. The result of competitive ELISA was expressed as B/B0, where B is the amount of antibody bound in the presence and B0 the amount in the absence of the competitor. Each point represents the mean of duplicate determinations.

The noncompetitive indirect ELISA was performed as described (6) with some modifications. Briefly, 50 µl of protein (0.01 mg/ml) was dispensed into wells and kept at 4°C overnight. The plate was then incubated with the monoclonal antibody (1 µg/ml), and the binding of antibody to the protein was evaluated by incubation with the anti-

mouse IgG antibody peroxidase-labeled. The data represent the mean of triplicate determinations.

Confirmation of N^{ε}-(hexanonyl)lysine formation in immunogen. The hydrolysate of the hexanonyl protein was dissolved in citrate buffer (pH 2.2) and applied to a JEOL JLC-500 auto amino acid analyzer. For liquid chromatography-mass spectrometry (LC-MS) analysis, the sample (the acetylated hydrolysate) was injected into a Develosil ODS-HG-5 column (4.6 x 150 mm, Nomura Chemical Co., Ltd., Aichi) and eluted with 0.1% acetic acid/CH_3CN (1/1) at a flow rate of 0.8 ml/min. The split eluent (9 vol.) was monitored at 280 nm by a UV-970 UV/Vis-detector (JASCO Co., Tokyo). The residual eluent (1 vol.) was directly applied to a PLATFORM II (VG Biotech, Tudor Toad Altrincham, WA) mass spectrometer using the electrospray ionization (positive) mode.

Animal care and exercise protocol. Twenty-eight female Sprague-Dawley rats (8 weeks of age), purchased from Japan SLC, Hamamatsu were used in the experiment. All procedures involving animals were approved by the experimental animal care committee of Nagoya Institute of Technology. The animals weighed 173–206 g. They were individually caged and housed at 23°C for 1 week with light from 5:00 a.m. to 5:00 p.m. and with a basal diet (commercial CE-2 powders, CLEA Japan, Ltd., Tokyo) and water *ad libitum*. Rats were randomly divided into a control group (N=14) and a trained group (N=14). The animals in the latter group were given a diets supplemented with 0.2 % eriocitrin *ad libitum*. The rats in each group were further divided into either a sedentary group (N=7) and a trained group (N=7). The rats in the trained group were exercised by running on a motor-driven treadmill designed for the mouse and rat (model III, Autome Kogyo, Tokyo) for 30 min/day at 5:00 p.m. for 3 weeks. During this period, the speed of the treadmill up a 6° incline was gradually increased from 20 to 30 m /min (20). The rats in the trained group were not exercised 24 h before they were sacrificed.

The diet for the eriocitrin group was prepared by mixing CE-2 powder with eriocitrin at 0.2%. The food intake was measured daily for one week before dissection. Body weight of the rats was measured every 3 days. Significant differences were not observed in the body weight gain and the daily food intake among all of the groups, indicating that exercise training and intake of the eriocitrin diet did not affect these parameters.

Tissue collection and biochemical analysis of oxidative stress. On the final day of the experiment, all of the rats were deprived of food for approximately 8 h in order to

adjust the metabolic conditions before dissection between 4:00 p.m. and 5:00 p.m. The rats were anesthetized with sodium pentobarbital. Gastrocnemius muscles were immediately removed, freeze-clamped at liquid nitrogen temperature, and stocked at –80°C until analyses. Rats were killed by exsanguination. The skeletal muscles obtained were homogenized in 10 volumes each of 50 mM sodium phosphate buffer (pH 7.4) at 4°C. The homogenate was centrifuged at 4,500 *g* for 15 min and the supernatant was obtained for the following biochemical measurement.

Statistical analyses. Values are represented mean ± SE (n=7). Statistical analysis was carried out by Two-Way ANOVA followed by Tukey's HSD test to identify significant differences. Differences were considered significant at $p<0.05$.

RESULTS

The confirmation of N^{ε}-(hexanonyl)lysine formation in proteins treated with hexanoic acid and carbodiimide

The hexanonyl KLH prepared was relatively insoluble; therefore, the confirmation was performed using hexanonyl BSA instead of the KLH. The modified BSA was hydrolyzed enzymatically, and the hydrolysates obtained were applied to an amino acid analyzer. A novel peak was observed between the position of valine and methionine, whose the elution time was the same as that of authentic HEL (chromatogram not shown). Furthermore, the hydrolysates were acetylated and then analyzed by LC-MS. The 287 $(M+H)^+$ ion of acetylated HEL was detected in the hydrolysates of hexanonylated BSA. The mass fragmentation pattern coincided with that obtained from authentic acetylated HEL (Fig. 1).

Preparation of monoclonal antibodies to HEL and these characterizations

The modified KLH was used for the immunogen, and the monoclonal antibodies were prepared as described in the Materials and Methods. Three clones (3C3, 5F12, 5H4) were obtained and the specificities were then examined. Competitive ELISA showed that these antibodies cross-reacted with Bz-Gly-N^{ε}-(hexanonyl)lysine but not its parent molecule (Fig. 2). Free HEL was also recognized by the antibodies to a similar extent (data not shown). The results suggest that the epitopes of monoclonal antibodies were the N^{ε}-(hexanonyl)lysine moiety. The effect of the length of the alkyl chain in the amide-type adduct (CH_3-$(CH_2)_n$-CO-NH-Lys; n=0–9) was also examined (Fig. 3). Although the 5H4 antibody reacted not only with n=4 (HEL) but also, to a lesser extent, with n=5 (N^{ε}-(heptanonyl)lysine), the monoclonal antibody had more specificity for the HEL moiety than did the polyclonal antibody. The other monoclonal antibodies (5F12 and 3C3) showed almost similar results. The reactivities of the 5H4 monoclonal antibody with aldehyde-modified proteins were examined. The aldehyde-modified BSAs were prepared by the incubation of BSA with commercially available aldehydes (malondialdehyde, 1-hexanal, and 2-hexenal) and synthetic 4-hydroxy-2-nonenal (6). The monoclonal antibody did not recognize these modified proteins (data not shown).

Reactivities of the antibody with peroxidized lipid-modified proteins

Using the 5H4 antibody, the formation of HEL by the treatment of BSA with peroxidized lipid was examined. As shown in Fig. 4, the incubation of the protein with 13-HPODE or 15-hydroperoxyeicosatetraenoic acid (15-HPETE) caused the formation of

immunoreactivity. The treatment of peroxidized linoleic acid, which had been prepared by the incubation with ascorbate/iron, significantly induced the HEL in the protein. HEL was also formed by the treatment with oxidized cardiolipin, which has esterified linoleic acid as the components. Ascorbate/iron-treated stearic acid (saturated fatty acid) and α-linolenic acid (ω-3 fatty acid) could not generate an antigen. These results are in good agreement with the previous results using the polyclonal antibody (6).

The protective effect of dietary flavonoid on HEL formation in the skeletal muscle of exercise-trained rats

The antibody was applied to *in vivo* experiments to examine the effects of exercise training and dietary eriocitrin on HEL formation in rat skeletal muscle. We evaluated the oxidative damage in the exercise-trained rats using the 5H4 monoclonal antibody to HEL. As shown in Fig. 5, we measured the levels of HEL in skeletal muscle in the sedentary or the exercise-trained rats. Dietary eriocitrin tended to decrease the HEL content in the skeletal muscle of sedentary rats. This effect of dietary eriocitrin was greater in trained rats; the content of HEL in skeletal muscle of trained rats was significantly lower in the eriocitrin diet group than in the control diet group. These results suggest dietary eriocitrin exerts a protective effect against exercise-induced oxidative stress.

DISCUSSION

Previously, the preparation of polyclonal antibodies against lipid hydroperoxide-modified proteins has been reported (3–5). Whereas the antibodies appear to be useful for the detection of lipid hydroperoxide-modified protein *in vivo* (21), the epitopes of these antibodies, including our antibodies prepared previously (4, 5), are still ambiguous. On the other hand, we have reported the identification of a novel lipid hydroperoxide-modified lysine residue, N^{ε}-(hexanonyl)lysine (HEL). Using the novel polyclonal antibodies specific to HEL, the presence of the HEL moiety in human atherosclerotic lesions was proven (6). The advantages of the anti-HEL antibody compared to other antibodies to lipid hydroperoxide-modified proteins are that the epitope of the antibody is strictly proven to be the HEL moiety, and that a monoclonal antibody is superior to a polyclonal antibody in the sense that the monoclonal antibody has a specific "mono" character. This is the first report to show the preparation of anti-HEL monoclonal antibody having a more specific reactivity toward the HEL moiety than the polyclonal antibodies (Fig. 3).

The biomarkers for oxidative stress have recently been of interest (22). The HEL moiety is generated from the reaction of lipid hydroperoxide with protein; harmful aldehydes cannot become the precursor (6). Based on those assumptions, the detection of HEL may result in the estimation of protein modification by lipid peroxidation in early stages. Whereas the precise mechanism of HEL formation is unknown, HEL should be a good biomarker for oxidative stress especially during the earlier stage of oxidative damage by lipid peroxidation than the advanced stage such as aldehyde formation occurrence.

It had reported that the administration of antioxidants, which are mixtures of α-tocopherol, ascorbic acid, butylated hydroxytoluene, and β-carotene, to sedentary rats and exercise-trained rats decreased dityrosine, one of the protein oxidation markers, in rat urine (23). We applied the monoclonal antibody to the evaluation of exercise-induced oxidative stress in rats. The administration of eriocitrin, which is one of components in lemon and lime fruits, to exercise-trained rat suppressed the formation of HEL, suggesting that (I) eriocitrin (including metabolites) directly trapped the oxygen radicals generated by its exercise and/or (II) eriocitrin (including metabolites) promoted the activation of defense mechanism such as the induction of antioxidative enzymes. The decrease in HEL content might be due to the progression of excretion (enhancement of turn over) of HEL from skeletal muscle by the activation of proteasomes (24). Further detailed studies are needed to explore both the mechanism for HEL formation and the

suppression mechanism by dietary eriocitrin.

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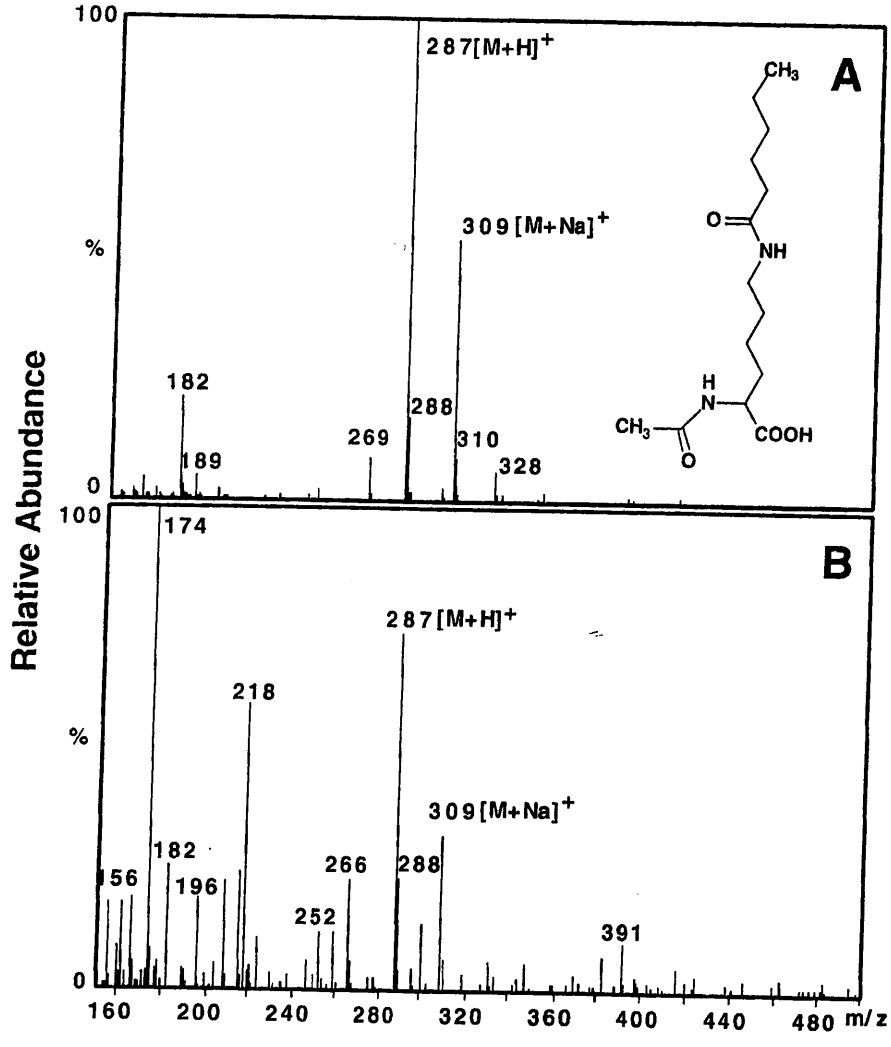

FIG. 1. Detection of the acetylated N^{ϵ}-(hexanonyl)lysine by LC-MS. The hexanonyl BSA was hydrolyzed enzymatically and derivatized as described under Materials and Methods. (A) Standard acetylated authentic HEL. (B) Acetylated hydrolysates of hexanonyl BSA. The structure of acetylated HEL is inserted into the A.

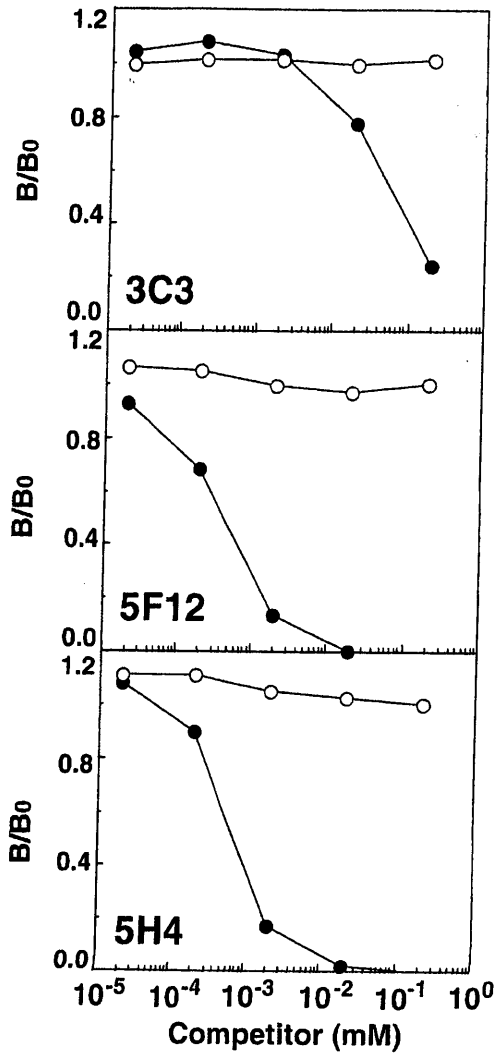

FIG. 2. The estimation of the epitope of monoclonal antibodies using the N^{ϵ}-(hexanonyl)lysine derivative. The indirect competitive ELISA by three monoclonal antibodies (3C3, 5H4, 5F12) was performed using hexanonylated BSA as the coating reagent. Open circle, Bz-Gly-Lys; Filled circle, Bz-Gly-N^{ϵ}-(hexanonyl)lysine.

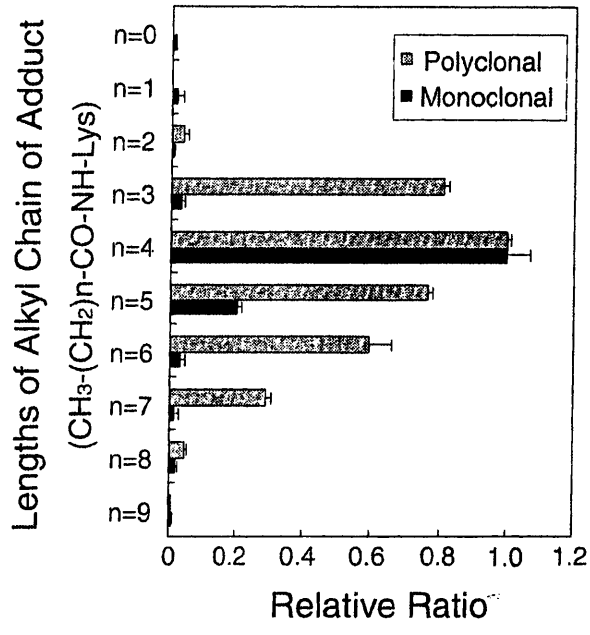

FIG. 3. Effect of alkyl chain lengths of amide-type adducts on the specificity. The various modified proteins having different lengths of the alkyl chain (CH_3–$(CH_2)_n$–CO–NH–, n = 0–9) were prepared as described (6). The estimation was done by solid-phase ELISA using the various modified BSAs as a coating reagent using the 5H4 monoclonal and polyclonal antibodies. The result is expressed as a relative ratio to the maximum value among the antigens by each antibody. Hexanonyl BSA, n = 4.

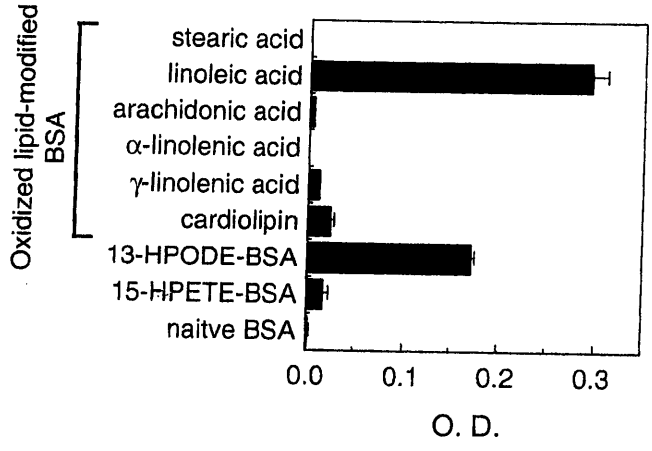

FIG. 4. Formation of HEL in proteins exposed to lipid peroxidation products. Stearic acid, linoleic acid, arachidonic acid, α-linolenic acid, γ-linolenic acid, and cardiolipin were oxidized by the incubation with ascorbate/iron. The reaction mixture was further incubated at 37°C for 3 days with lipid-free BSA in 0.1 M phosphate buffer (pH 7.4) and the modified BSA was then obtained. 13-HPODE and 15-HPETE were prepared by incubation of linoleic acid and arachidonic acid, respectively, with soybean lipooxygenase. The lipid hydroperoxides obtained were incubated with lipid-free BSA at 37°C for 3 days in the phosphate buffer and the lipid hydroperoxide-modified BSAs were then obtained. The formation of HEL in the peroxidized lipid-modified BSA was estimated by ELISA using the 5H4 antibody.

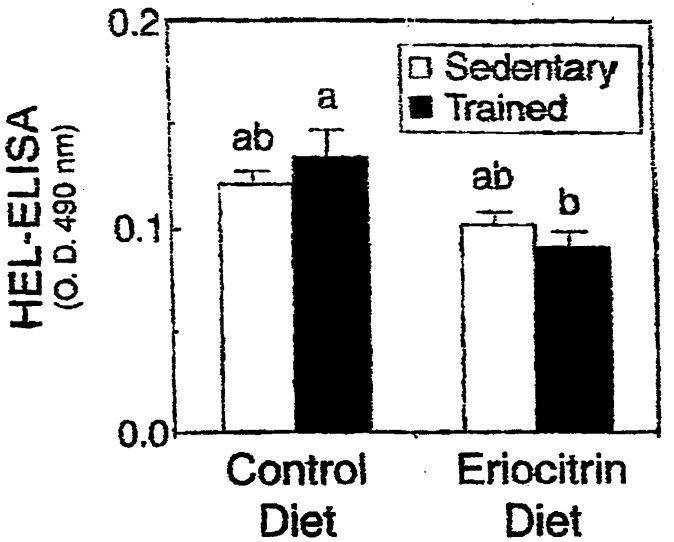

FIG. 5. The level of HEL of oxidative modified protein in skeletal muscle on the sedentary/exercise-induced rats with/without eriocitrin diet. Values are means ± SE of 7 rats per group. The statistical significant of the results among training exercise rat groups was analyzed by analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Values within the same row that do not share a common superscript letter are significantly different at $P < 0.05$.

ジチロシンに特異的なモノクローナル抗体の作製と応用

ABSTRACT

Dityrosine is one of the specific markers for protein oxidation. The detection of dityrosine in protein molecules may become a good biomarker of oxidative protein modification. We planned for the preparation of an antibody specific for protein dityrosine using a dimer of 3-*p*-(hydroxyphenyl)propionic acid (di-HP) as a hapten. Three clones (A8, G6, and 1C3) were obtained, and the antibody from A8 clone reacted with the di-HP-conjugated protein but not with the dityrosine conjugate. The others (G6 and 1C3 clones) recognized both the di-HP and dityrosine conjugates. The latter two obtained monoclonal antibodies recognized H_2O_2/Cu-oxidized lens proteins and BSA. However, the net amount of dityrosine, determined by HPLC, in the oxidized proteins did not coincide with the immunoreactivity with the antibody, suggesting that dityrosine in oxidized BSA might be buried in the protein molecule. Using the obtained monoclonal antibody (G6), the immunopositive staining of atherosclerotic lesions in Apo E-deficient mice was confirmed by immunohistchemical technique.

Key words: dityrosine, monoclonal antibody, oxidation, atherosclerosis, Apo E-deficient mouse.

The participation of oxidative stress in many diseases including aging processes has been assumed. The estimation of the levels of oxidative modification using biomarkers for oxidatively modified biomolecules may become a useful tool for evaluation of the "oxidative" status in our body. Among the oxidative modifications of biomolecules, protein carbonyl has often been used for the quantification of protein oxidation. The increase in protein carbonyl during aging and with some diseases has been reported using the detection of the carbonyl moiety by dinitrophenylhydrazine (1–4). The carbonyl measurement as the total oxidative modification marker (like thiobarbituric acid methods in the estimation of lipid peroxidation) might be superior to some more specific methods such as the direct measurements of oxidized amino acids (5–7). However, the carbonyl is derived from not only "direct" protein oxidation but also from secondary reactions such as non-enzymatic glycation and lipid peroxidation (8, 9).

Dityrosine is a fluorescent dimer of tyrosine and is formed by reactive oxygen species (10), ultraviolet irradiation (11), and peroxidases (12, 13). The isolation and identification in connective tissue proteins such as collagen in tendons or the skin, and elastin in the aorta of chick embryos have been reported (14–17). The presence of dityrosine bridges in thyroglobulin, the prothyroid hormone, has also been confirmed (18–20). The detection of dityrosine in atherosclerotic lesions was also reported (21, 22).

Antibodies to oxidative stress markers have been widely used, and the immunological methods has much advantages on the study for localization of the markers and its convenience, whereas these methods are semi-quantitative. We have focused on dityrosine as one of the biomarkers for oxidative protein damage, and the specific polyclonal antibody has already been prepared (23). However, the polyclonal antibody was not suitable for the estimation of dityrosine buried in protein because of its preferential specificity for free dityrosine more than for peptidyl dityrosine. We have improved the preparation protocol to obtain a monoclonal antibody recognizing protein dityrosine using a novel immunogen. In this report, the specificity of three monoclonal antibodies obtained has been shown. Using the antibody, the formation of protein dityrosine in atherosclerotic lesions of Apo E-deficient mice has been estimated.

MATERIALS AND METHODS

Materials

Dimethylformamide, *N*-hydroxysucciniimide, hydrogen peroxide (H_2O_2, 30%), and 3-(*p*-hydroxyphenyl)propionic acid (HP) [2] were obtained from WAKO. Horseradish peroxidase (HRP), *Arthromyces* peroxidase, catalase, lens proteins (from bovine eye lens: water soluble), and lipid-free bovine serum albumin (BSA) were purchased from Sigma Chemical Co. Keyhole limpet hemocyanin (KLH) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were purchased from Pierce.

Preparation of Immunogen

The dimer of 3-(*p*-hydroxyphenyl)propionic acid (HP) was prepared by incubation of HP with HRP in the presence of H_2O_2. Briefly, HP (0.17 g), HRP (3 mg), and 30% H_2O_2 (120 µl) in 200 ml of 0.01M borate buffer (pH 9.0) were incubated at 37°C for 2 h. The reaction was terminated by the addition of catalase 5 µl (20,000 units) and then further incubated for 15 min. The product was concentrated by freeze-drying, and dissolved in water, and then applied to an HW-40 gel filtration open column, which had been equilibrated with water. The fractions having the fluorescence (ex. 300 nm/em. 400 nm) were collected and concentrated. Aliquots of the fractions were simultaneously analyzed by reversed phase high performance liquid chromatography (HPLC), and it was confirmed that a single peak (detected at UV 280 nm), which has the fluorescence nature of the dihydroxyphenyl moiety, was included in the fractions. The liquid chromatography-mass spectrometry (LC-MS) analysis of the fraction revealed that the product showed 329 $(M-H)^-$ as the parent ion. Based on these results, the product was identified as a dimer of 3-(*p*-hydroxyphenyl)propionic acid, named di-HP. The HPLC and LC-MS analyses were done as described in the section below .

The obtained dimer (di-HP) was conjugated with KLH and BSA using EDC and *N*-hydroxysucciniimide. In brief, the dimer (16 mg), EDC (18 mg) and *N*-hydroxysucciniimide (20 mg) were dissolved in 1.6 ml of dimethylformamide and reacted at room temperature overnight with stirring. The reaction mixture was divided equally. One part was mixed with 30 mg of lipid-free BSA in 3.8 ml of phosphate buffer (pH 7.4), and the other was mixed with 20 mg of KLH in 3.8 ml of the phosphate buffer at room temperature with stirring for 4 h. The modified proteins (di-HP-KLH and di-HP-BSA) were dialyzed against water at 4°C for 2 days with several exchanges of water. The concentration of proteins was determined by BCA assay (Pierce), and the concentration was adjusted to 1 mg/ml with phosphate-buffered saline (PBS) and then stored at –70°C before use.

HPLC and LC-MS of di-HP

The sample was injected into a Develosil ODS-HG-5 column (4.6 x 150 mm) and eluted with 0.1% acetic acid/CH_3CN (1/1) at a flow rate of 0.8 ml/min. The elution was monitored by a UV/Vis-detector (280 nm) and a fluorescence detector (ex. 300 nm/em. 400 nm). For the LC-MS, the separation by HPLC was performed under the same conditions. The detection was performed by a PLATFORM II (VG Biotech) mass spectrometer using the electrospray ionization (positive) mode.

Preparation of chemically modified proteins

Dityrosine-conjugated BSA (DT-G-BSA) was prepared by incubation of lipid free BSA and dityrosine with glutaraldehyde as described (23). Dityrosine was synthesized enzymatically and purified as described (11).

Immunization and antibody preparation

Balb/c mice were primed intraperitoneally with the modified KLH (di-HP-KLH, 75 μg/mouse) emulsified in Freund's complete adjuvant. Mice were boosted twice at a 2-week interval. After 8 weeks, the animals received an intraperitoneal final injection of the antigen without adjuvant. The production and selection of hybridomas were carried out by the method of Kohler and Milstein (24) with a slight modification. The (positive) clones were selected using the di-HP-BSA and/or dityrosine-conjugated BSA (DT-G-BSA) as antigen for enzyme-linked immunosorbent assay (ELISA). The hybridomas obtained were injected intraperitoneally into Balb/c mice, which had been injected with 0.5 ml of pristane (2, 6, 10, 14-tetramethylpentadecane) one week before and about 7 – 10 days later, the ascites were collected by laparotomy . The types of the antibodies were estimated by antigen-mediated ELISA using a Mouse Isotyping Kit (Sigma) according to the manufacturer's recommendations. Partially purified antibodies were prepared using ammonium sulfate fractionation and used for experiments.

Oxidative modification of proteins with H2O2/Cu

Proteins (0.5 mg/ml) were incubated with H_2O_2 (0–10 mM) and $CuSO_4$ (0–0.05 mM) in a 0.1 M phosphate buffer (pH 7.4) at 37°C for 0 – 24 h. The reaction was terminated by adding 0.1 mM ethylenediaminetetraacetic acid (EDTA). The reaction mixture was stored at –20°C or –70°C until use.

Dityrosine measurement by HPLC

Proteins were dialyzed against water and then used for acid hydrolysis. The dityrosine in the hydrolysates was measured by HPLC-fluorescence detector as described (11).

Enzyme-linked immunosorbent assay (ELISA)

The indirect ELISA was performed as described: Briefly, for the time-course experiments, 50 µl of modified proteins (0.01 mg/ml in PBS containing 0.1 mM EDTA was dispensed into wells and kept at 4°C overnight. After the coating, the plate was washed with PBS containing 0.25% Tween 20 (TPBS) and water, and 200 µl of 2% blocking agent (Block Ace, Dainihon Seiyaku, Osaka, Japan) was then added and further incubated for 1 h at 37°C. The plate was then incubated with the monoclonal antibody (0.1 – 0.2 µg/ml TPBS) for 2 h at 37°C, and the binding of antibody to the modified protein was evaluated by incubation with an anti-mouse IgG antibody peroxidase-labeled (1/5000). The color development was performed by reaction of the conjugated peroxidase with *o*-phenylenediamine and H_2O_2. The data represent the mean of triplicate determination.

The cross-reactivity with antibody was also investigated by indirect competitive ELISA: As a coating agent, 50 µl of di-HP-BSA (0.1 – 0.5 µg/ml PBS) was pipetted onto wells and kept at 4°C overnight. At the same time, 50 µl of antibody (0.05 – 0.1 µg/ml TPBS) and 50 µl of sample were mixed in an Eppendorf tube and reacted at 4°C overnight. The plate was washed, blocked, and 90 µl of the reacted solution was then pipetted onto a well. The binding of the residual antibody on coated modified BSA was estimated as described above. The result of competitive ELISA was expressed as B/Bo, where B is the amount of antibody bound in the presence and Bo the amount in the absence of the competitor. Each point represents the mean of duplicate determinations.

Animals

Purchased from ??????? care ????????

Immunohistochemical analysis

Tissue sections (5 µm) were prepared from paraffin embedded aortic arch of 6-month-old male Apo E-deficient mice fed normal chow. Sections were immunostained with Vectrastain ABC-AP (alkaline phosphatase) commerical kit (Funakosi, Tokyo, Japan). Sections were incubated with normal serum in PBS for 20 min to block nonspecific binding before staining and then with the G6 monoclonal antibody (1:1000 dilution) or 5% normal mouse serum IgG instead of the primary antibody as the negative control. Immunosttaining was performed with biotinic affinity refined immunoglobulin (1:200 dilution) as the second antibody and with the reagent avidin DH plus biotinic alkaline phosphatase H in a ratio of 1:1 as the enzymatic marker and with Vector Red commerical kit as chromogen.

RESULTS

Preparation of a novel immunogen and the reactivity with polyclonal antibody

We have already reported the preparation of polyclonal antibody to dityrosine using dityrosine-conjugated keyhole limpet hemocyanin (23). The polyclonal antibody recognized free dityrosine but not other tyrosine-related compounds. However, weak binding ability to protein dityrosine was assumed by comparison of tripeptidyl dityrosine with free dityrosine. The result suggested that free dityrosine was probably attached to the surface of carrier protein like protein tyrosylation. To prepare the antibody recognizing protein dityrosine (intra- and intermolecular protein-bound dityrosine), a dityrosine-like compound, the dimer of 3-(*p*-hydroxyphenyl)propionic acid (di-HP), was used as a hapten because of effective conjugation. The 3-(*p*-hydroxyphenyl)propionic acid (HP) was treated with horseradish peroxidase (HRP) in the presence of H_2O_2, and the formed dimer (di-HP) was then conjugated with KLH and used for the immunogen.

At first, we have examined the reactivity of polyclonal antibody to di-HP conjugated KLH (immunogen) and its analog (di-HP-BSA) to check the antigen preparation. The polyclonal antibody to dityrosine could also recognize the novel synthetic conjugates (data not shown). The results suggested that the epitopes of polyclonal antibody resembled the di-HP moiety.

The production of monoclonal antibodies and competitive ELISA with dityrosine conjugated protein

The novel immunogen, modified KLH, was injected into mice, and monoclonal antibodies were prepared by fusion of myeloma cells with spleen cells using polyethylene glycol according to the standard protocol. The generation of the antibody to the dihydroxyphenyl moiety was estimated by ELISA using both DT-G-BSA and di-HP-BSA. As a control, the mybridoma (named A8 clone), which generates the antibody to di-HP-BSA but not DT-G-BSA, was also selected. After all, three clones (A8, G6, and 1C3) were obtained. The results of competitive ELISA using these clones are shown in Fig. 1. The antibody from A8 clone reacted with the di-HP-conjugated protein but not with the dityrosine conjugate (Fig. 1*A*). The others (G6 and 1C3 clones) recognized both the di-HP and dityrosine conjugates (Fig. 1*B* & *C*).

Estimation of epitopes by competitive ELISA using low molecular weight compounds as competitors

The monoclonal antibodies (G6 and 1C3) reacts with free dityrosine, whereas other tyrosine related compounds, such as tyrosine, phenylalanine, 3-nitrotyrosine, and 3-chlorotyrosine, could not be recognized by the antibody (data not shown). The antibody reacted with dityrosine in the dimer of Thr-Tyr-Ser rather than free dityrosine,

suggesting preferential recognition of the antibody to protein dityrosine compared to free dityrosine (Fig. 2).

Application of the antibodies to oxidatively modified proteins

Next, we examined the formation of immunoreactivity with H_2O_2/Cu-oxidized lens protein and BSA. The results were shown by comparison of the positive control (DT-G-BSA 0.5 μg/ml) as a relative ratio. The formation of the immunoreactivity (1C3 clone) was increased with increasing H_2O_2 concentrations (Fig. 3). The omission of copper ion in the system caused the disappearance of the reactivity. In addition, the antibody secreted from the G6 clone was also reacted with the oxidized proteins similar to that from the 1C3 clone. The antibody from the A8 clone (di-HP specific) could not recognize these oxidatively-modified proteins.

Dityrosine formation of proteins exposed to H_2O_2/Cu

Fig. 4 showed that the difference in immunoreactivity between oxidized lens protein and BSA. Next, the immunoreactivity of the 1C3 antibody with oxidized proteins was compared with the dityrosine contents, determined by HPLC connected to a fluorescence detector. From a preliminary study, we had found that apotransferrin was also a good antigen precursor. Therefore, three types of transferrins (apo, partially iron-saturated, and holo (iron-saturated) types), obtained commercially, were also used for substrates, and the differences of immunogenicity between Fe contents have also been investigated. As shown in Fig. 4*A*, considerable binding to oxidized apotransferrin as well as oxidized lens protein was observed by ELISA. Control proteins (incubated without H_2O_2/Cu) could not be recognized by the antibody. Oxidized BSA could be hardly recognized by the antibody. The modified proteins were hydrolyzed by 6 N HCl and the hydrolysates were applied to the HPLC for chemical quantitation of dityrosine. Dityrosine was formed from oxidized lens protein (1377 ± 12 pmol/mg protein) but not from the native one (control, 17 ± 2 pmol/mg protein). However, the profile of dityrosine content (Fig. 4*B*) was not consistent with the profile of immunoreactivity (Fig. 4*A*), especially on BSA. These differences are possibly derived from the structure of the protein which possibly has a direct effect on antibody recognition. For example, dityrosine in oxidized BSA might be buried in the protein molecule and, therefore, the antibody could hardly react with or contacted the protein dityrosine.

Immunohistochemical study

The detection of dityrosine in atherosclerotic lesion was already reported using chemical methods by HPLC or gas chromatography-mass spectrometry (21, 22). Therefore, we examined the immunohistochemical staining of dityrosine using the monoclonal antibody in atherosclerotic lesion of Apo E-deficient mice. As shown in Fig.

5A (arrow) and 5C, the positive staining in the fatty streak was observed.

NORMAL MICE

DISCUSSION

To prepare the antibody specific to the dihydroxyphenyl moiety (common structure of free and protein-buried dityrosine), the antibody recognizing dityrosine in protein (peptidyl dityrosine) was prepared using a novel immunogen having a dityrosine-like hapten (dimer of 3-(*p*-hydroxyphenyl)propionic acid). Among three clones obtained, two clones (G6 and 1C3) generated the antibodies recognizing free dityrosine, dityrosine-conjugated protein (DT-G-BSA), and H_2O_2/Cu-oxidized proteins. The immunoreactivity of the monoclonal antibodies with oxidized lens protein was significantly higher than that of oxidized BSA, whereas the dityrosine contents determined by HPLC were almost similar among the two proteins. The reason is not known but the conformation (accessibility) may account for the differences.

Dityrosine is formed via tyrosyl radical intermediates. The tyrosyl radical can cause lipid peroxidation via radical reactions (H-abstraction of the lipid moiety) (25) and the participation of myeloperoxidase in dityrosine formation has been suggested (21, 26). Though dityrosine was already detected from LDL isolated from atherosclerotic lesions (21, 22), In this report, the localization of dityrosine in the fatty streak of atherosclerotic plaque in Apo E-deficient mice was visually revealed by an immunohistochemical method using the novel monoclonal antibody (developed using avidin-biotin-alkali phosphatase complex). *In additon, the positive staining was also observed using avidin-biotin-peroxidase development (data not shown). Because peroxidases (12, 13) can generate dityrosine, alkalil phosphatase was used for the development to avoid the possibility of contribution of nternal peroxidase activity to the staining.* The plausible contribution of myeloperoxidase to the formation of dityrosine in atherosclerotic plaque can be confirmed in part by evidence of the presence of active myeloperoxidase in the lesion (26).

It has been reported that the caloric restriction attenuated dityrosine contents in cardiac and skeletal muscles of mice has been reported (27). In these way, the detection/quantification of dityrosine would become a biomarker of 'pure' protein oxidation. 'Pure' means that the formation of dityrosine is not generated by lipid-derived and glycation-derived aldehydes. The nonimmunochemical methods such as gas chromatography-mass spectrometry or HPLC-electrochemical detection are suitable for the "correct" (net) quantitation of dityrosine, whereas these approaches for detection of dityrosine require several steps of sample preparation. The immunochemical methods should be useful roles for dityrosine detection in cells and tissues with simple procedures.

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Figure legends

FIG. 1. Competitive ELISA of synthetic hapten- and dityrosine-conjugated proteins. The hapten (a dimer of 3-(*p*-hydroxyphenyl)propionic acid) was conjugated with BSA using carbodiimide and the conjugate (di-HP-KLH) was then dialyzed. Dityrosine-conjugated BSA (DT-G-BSA) was prepared by incubation of lipid-free BSA and dityrosine with glutaraldehyde. The cross-reactivity of antibodies ((*A*) A8 clone, (*B*) G6 clone, and (*C*) 1C3 clone) with the proteins was investigated by indirect competitive ELISA using di-HP-BSA as a coating agent. The sample and antibody were reacted overnight in a tube and then added to the ELISA plate, which had been blocked. The competition between the sample and coated di-HP-BSA was evaluated by incubation of the plate with anti-mouse IgG antibody as described in Materials and Methods.

FIG. 2. The antibody recognized peptidyl dityrosine rather than free dityrosine. The dimer of Thr-Tyr-Ser (Di-TYS) was prepared using *Arthromyces* peroxidases in the presence of H_2O_2 and Thr-Tyr-Ser as described (23). The presence of the dityrosine moiety in the dimer was confirmed by reversed phase HPLC connected to a fluorescence detector, following acid hydrolysis of the isolated Di-TYS. The ELISA using 1C3 clone was performed as described in the legend to Fig. 1. The antibody from G6 clone showed similar results (data not shown).

FIG. 3. The reactivity of lens protein and BSA with the 1C3 antibody was increased with increasing H_2O_2 concentration. The proteins were incubated with 0.05 mM $CuSO_4$ at various concentrations of H_2O_2 (0 – 10 mM). The reaction was terminated by the addition of 1 mM ethylenediaminetetraacetic acid (EDTA) and was used for the assay. The ability for binding was estimated by indirect noncompetitive ELISA. Protein (50 µl, 0.01 mg/ml in PBS/EDTA) was coated on a well and the plate was kept overnight at 4°C. The plate was washed and then blocked by a blocking agent. The antibody (1C3) was added to the plate and incubated for 2 h at 37°C. The binding of the antibody to the coated sample was determined by incubation of peroxidase-conjugated antibody to mouse IgG and development with H_2O_2 and *o*-phenylenediamine. The data was expressed by comparison with positive controls (DT-G-BSA 0.5 µg/ml) as a relative ratio.

FIG. 4. Comparison between the immunoreactivity formation and dityrosine contents by treatment of proteins with H_2O_2/Cu. The reaction with H_2O_2/Cu was performed as described in Materials ans Methods. As a control, proteins were also incubated without H_2O_2/Cu. After 4-h incubation, the reaction was terminated and the proteins were dialyzed against water at 4°C for 2 days with several exchanges of water. The concentration of protein was measured and used for the following experiments. (*A*) The

formation of immunoreactivity during oxidation of protein. Protein was diluted with PBS at 0.01 mg/ml and applied to ELISA. The ELISA was done as described in the legend to Fig. 3. (*B*) The formation of 'net' dityrosine in hydrolysates of the protein during incubation with/without H_2O_2/Cu. An aliquot of the dialyzed solution was freeze-dried and then hydrolyzed by 6 N HCl in vacuo at 105°C for 24 h. The hydrolysates were dried and dissolved in 0.01 N HCl and then applied to reversed phase HPLC connected to a fluorescence detector. The quantitation of dityrosine was performed by comparison with synthetic dityrosine. Apotransferrin, transferrin and holotransferrin mean Fe-poor, partially Fe-saturated, and Fe-saturated transferrin, respectively.

FIG. 5. Photomicrographs showing positive immunostaining of protein dityrosine in the sections of aortic arch of a 6-month-old male Apo E-deficient mice fed normal chow. *A & C*, sections stained with the G6 antibody (1:1000 dilution), specific epitopes recognized by the primary antibody are indicated by the red color in the fatty streak. *B*, no staining was observed in the section stained with 5% normal mouse serum IgG instead of the primary antibody as the negative control. Arrow in the A (magnification x 40) indicates the positive staining at a fatty streak and the position is enlarged x 200 (C). (*A & B* x 40; *C* x 200)

Fig. 1

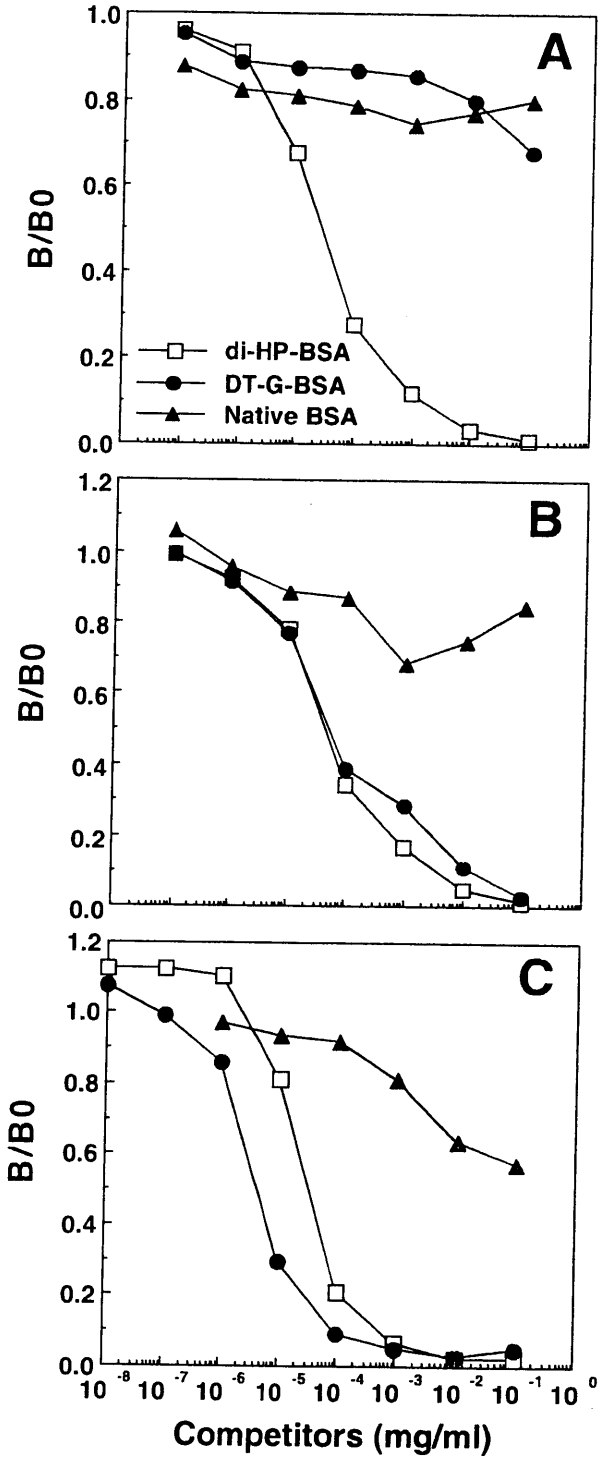

A
B
C
B/B0
1.0
0.8
0.6
0.4
0.2
0.0
1.2
di-HP-BSA
DT-G-BSA
Native BSA
10 -8 10 -7 10 -6 10 -5 10 -4 10 -3 10 -2 10 -1 10 0
Competitors (mg/ml)

Fig. 2

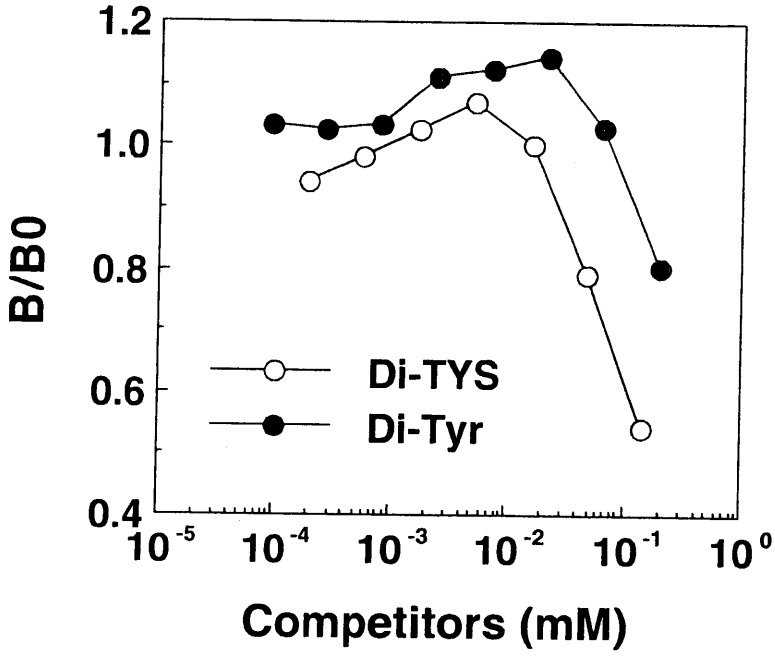

1.2
1.0
0.8
0.6
0.4
B/B0
Di-TYS
Di-Tyr
10 -5
10 -4
10 -3
10 -2
10 -1
10 0
Competitors (mM)

Fig. 3

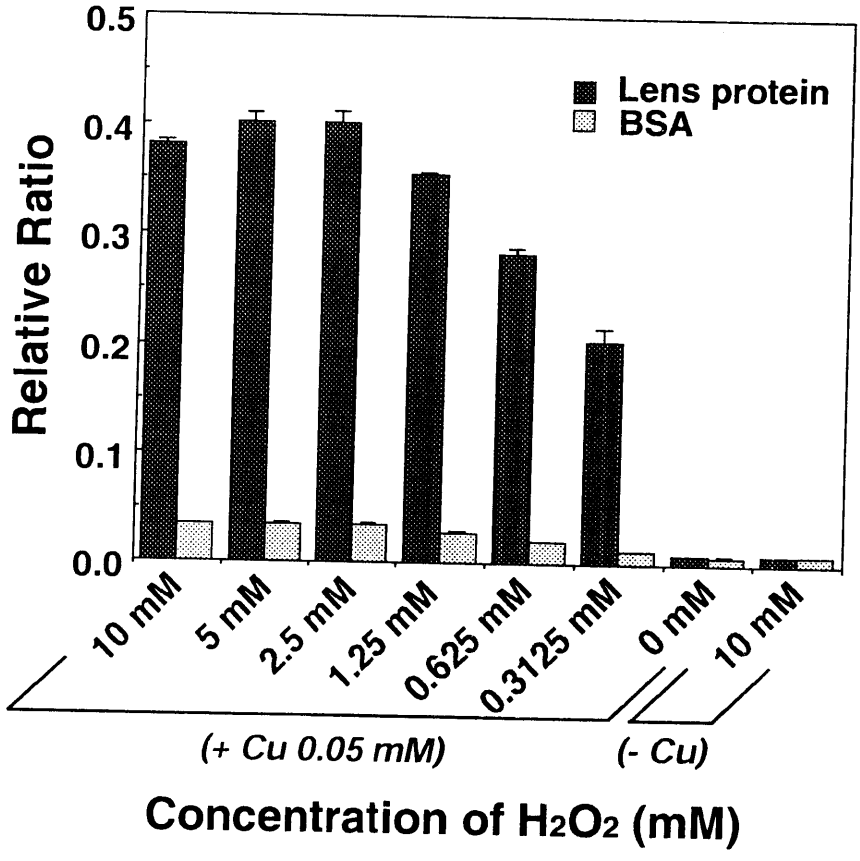
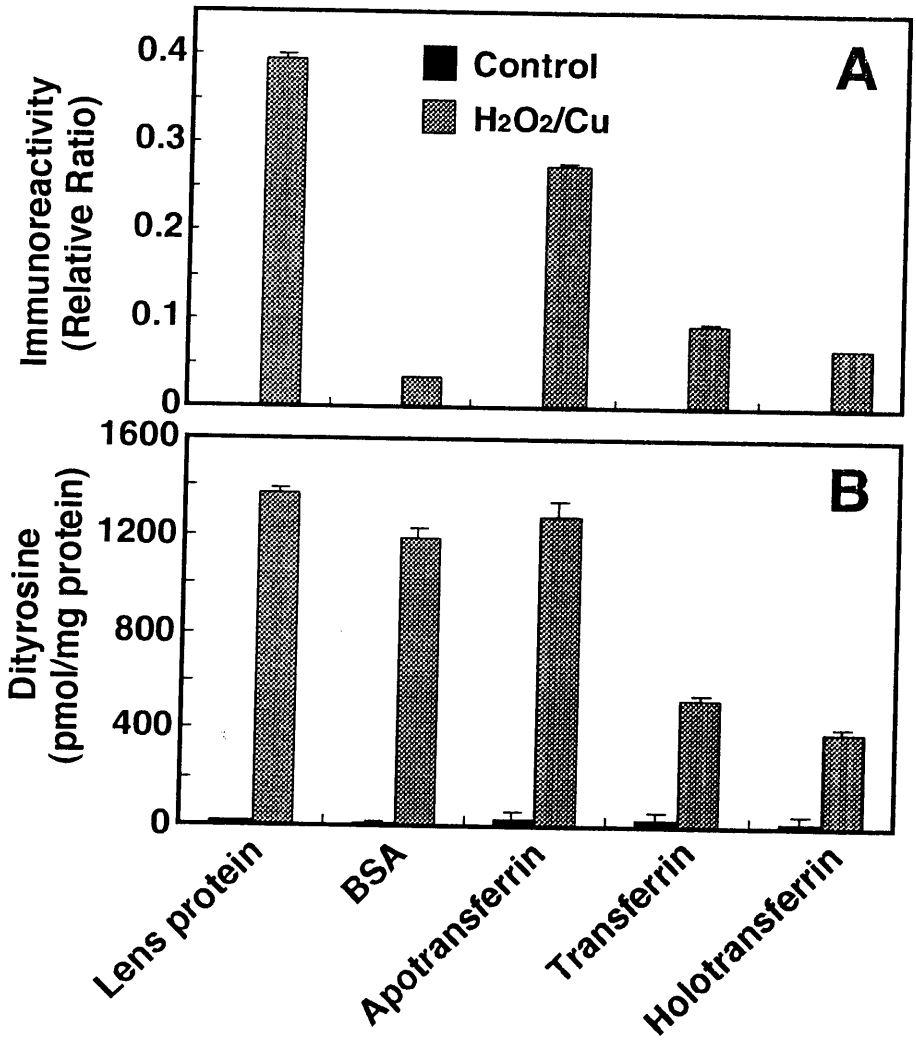

0.5
0.4
0.3
0.2
0.1
0.0
Relative Ratio
Lens protein
BSA
10 mM
5 mM
2.5 mM
1.25 mM
0.625 mM
0.3125 mM
0 mM
10 mM
(+ Cu 0.05 mM)
(- Cu)
Concentration of H2O2 (mM)

Fig. 4


Immunoreactivity
(Relative Ratio)
0.4
0.3
0.2
0.1
0
Control
H2O2/Cu
A
Dityrosine
(pmol/mg protein)
1600
1200
800
400
0
B
Lens protein
BSA
Apotransferrin
Transferrin
Holotransferrin

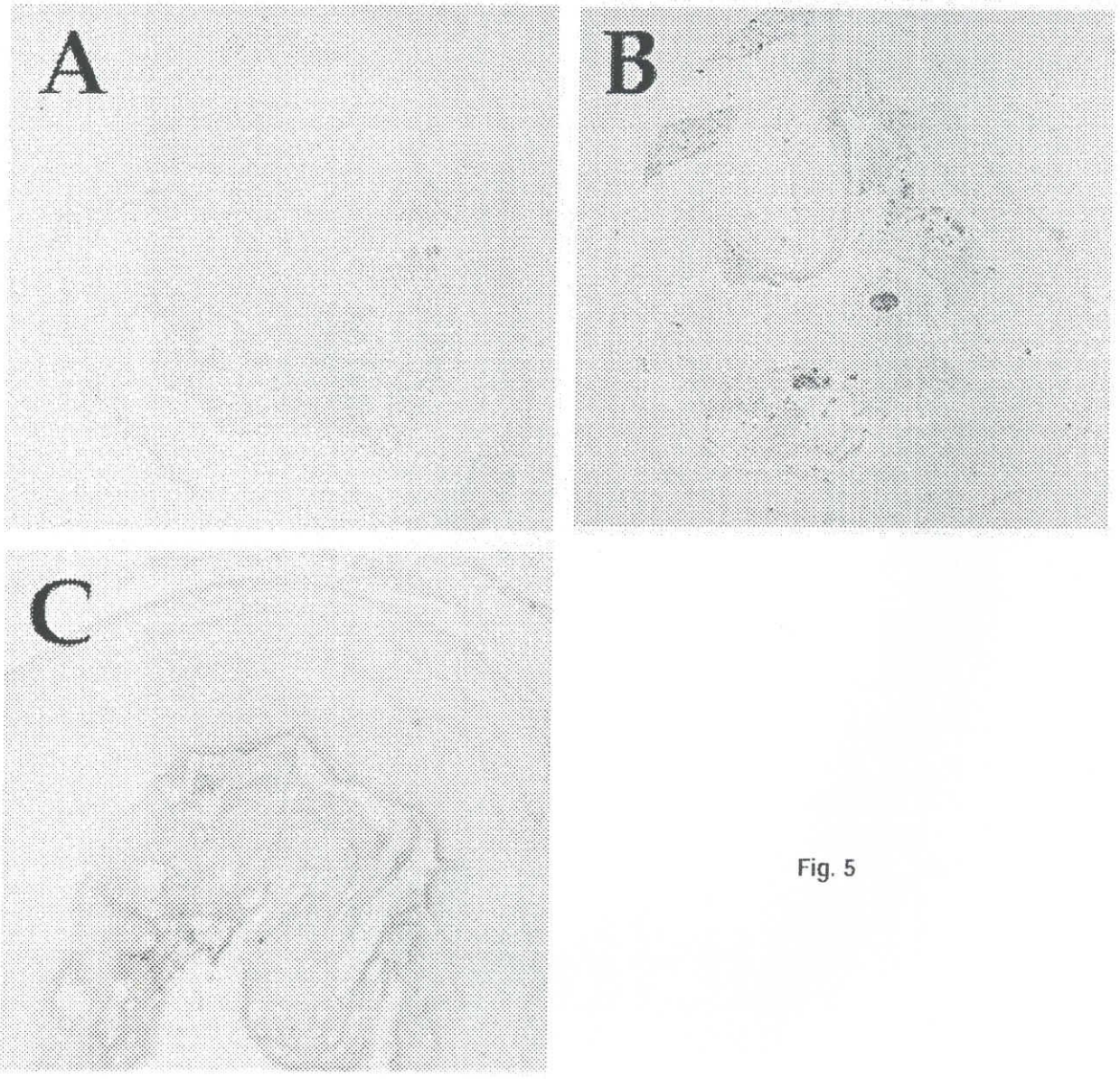


Fig. 5

2．　II型糖尿病発祥モデル、Akita mouse における酸化ストレスの関与

糖尿病における高血糖状態は、タンパク質の糖化反応、ポリオール代謝やグルコース自動酸化を亢進し、その結果生成した活性酸素が動脈硬化、腎障害、糖尿病性白内障などの原因となると考えられている。本研究では明らかな高血糖を呈する２型糖尿病モデルである Akita mouse の酸化ストレス状態を解析することを目的とし、血液および組織の脂質過酸化やタンパク質の酸化障害を検討した。方法は、２型糖尿病発症モデルである Akita mouse（雄）およびその対照系統（C57BL/6）マウス（雄）を５週齢から８週間飼育した後、屠殺して血清、腎臓、肝臓を得た。得られた腎臓、肝臓はホモジナイズして、その上清を測定試料とした。脂質過酸化度測定のために、TBARS の定量を行うとともに、脂質過酸化初期反応生成物であるリノール酸１３－ヒドロペルオキシドとリジンとの付加体である N^{ϵ}-(hexanonyl)lysine (HEL)[2] の検出を試みた。さらに、タンパク質の酸化障害を検討するために、試料中の dityrosine (DT)の検出を試みた。その結果、（１）全飼育期間を通じて、Akita mouse は 350 mg/dl 以上の高血糖を示し、対照系統では正常値が確認された。（２）Akita mouse を用いて、TBARS を指標とした脂質過酸化反応の進行の検討を行った結果、肝臓と腎臓では有意な変化は見られなかったが、Akita mouse の血清中の TBARS が有意な高値を示した。また、抗 HEL 抗体を用いた ELISA 法により、Akita mouse の腎臓において HEL が有意に増加していることが明らかとなった。　（３）抗ジチロシン抗体を用いた ELISA 法にて、Akita mouse の腎臓において DT が有意に増加していた。

以上の結果より、Akita mouse の腎臓では、脂質過酸化初期反応生成物の蓄積とタンパク質の酸化傷害の亢進が示された。このことは高血糖状態が生体内での酸化ストレスを亢進していることを示唆するものである。この内容は、オリジナル論文として準備できたので、ここに紹介したい

Footnotes

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Abbreviations

BSA: bovine serum albumin
DT: dityrosine
ELISA: enzyme-linked immunosorbant assay
HEL-BSA: hexanonyl BSA
HEL: N^{ε} - (hexanonyl) lysine
13-HPODE: 13-hydroperoxyoctadecadienoic acid
8-OHdG: 8-hydroxydeoxyguanosine
HNE: 4-hydroxy-2-nonenal
PBS: phosphate-buffered saline
TBARS: thiobarbituric acid reactive substances
ROS: reactive oxigen spices
NIDDM: non-insulin dependent diabetes mellitus
KLH: keyhole limpet hemocyanin
EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
NHS: N-hydroxysuccinimide
HP:3-(p-hydroxyphenyl)propionic acid
ABC: avidin-biotin complex

Abstract

Using the new biomarkers for oxidative damage, we tried to estimate whether hyperglycemia causes oxidative stress in Akita mice, an animal model for type 2 diabetes at an early age. Five-week-old male Akita mice and the control normoglycemic mice (C57BL/6) were maintained until 13 weeks of age. After 8 weeks of feeding, the tissues (liver and kidneys) of both strains were obtained and the level of N^{ϵ}- (hexanonyl) lysine (HEL) and dityrosine (DT) were measured, which are related with respect to lipid peroxide-derived protein covalent modification and protein cross-linking. The levels of HEL and DT in the kidneys of the Akita mice were significantly increased compared with the control mice without the accumulation of thiobarbituric acid reactive substances (TBARS) and the 4-hydroxy-2-nonenal (HNE) modified protein. Immunopositive staining was clearly observed in the kidneys of the Akita mice using anti-HEL antibody or anti-DT antibody. These results suggest that hyperglycemia in Akita mice induces an increase in these markers and oxidative stress occurs in the kidneys at an early age.

1. Introduction

The excessive production of reactive oxygen species (ROS) are thought to cause damage *in vivo*. This damage is implicated in the process of many diseases. To evaluate the extent of oxidative stress, some oxidative stress markers have been developed and recognized as useful markers for the estimation of oxidative stress [1]. For example, 8-hydroxydeoxyguanosine (8-OHdG), which is produced by the oxidation of DNA, has been commonly used for one of the markers of oxidative DNA damage [2, 3]. HNE (4-hydroxy-2-nonenal) is one of the typical aldehyde products, and has also been used for the marker of lipid peroxidation[4]. Recently, N^{ε} - (hexanonyl) lysine (HEL) and dityrosine (DT) , which are the oxidative stress markers, have been developed and detected in various diseases [5, 6].

Lipid hydroperoxides are formed during the initial stage of lipid peroxidation. During the process of membrane lipid peroxidation, various aldehydes are subsequently formed as the final products after the lipid hydroperoxides were broken down. HNE is formed by the lipid peroxidation of the omega-6 unsaturated fatty acid as lipid peroxidation advanced end products and HNE-modified biomolecules are accumulated in some tissues for various diseases [7]. Kato et al. reported that HEL is formed by the reaction of 13-hydroperoxyoctadecadienoic acid, a model of the lipid hydroperoxides, with lysine residue during the early stage of lipid peroxidation. The anti-HEL antibody was prepared and the presence of HEL in oxidized LDL was confirmed[5]. Furthermore, positive staining was observed in human atherosclerotic lesions using an immunohistochemical technique [5]. The results indicated that HEL is an initial marker for oxidative damage of biological molecules *in vivo*[5].

The oxidation of tyrosine generates a tyrosyl radical, and DT is then formed by the reaction of two tyrosyl radicals. DT is formed by ROS [8, 9], enzymatic reactions [10, 11], UV irradiation [12] and lipid peroxidation [13]. The anti-DT antibody was developed and it demonstrated that DT can be detected in lipofuscin from the aged human brain [6], suggesting that DT may become a useful marker for the estimation of protein cross-linking.

Hyperglycemia causes the autoxidation of the glucose [14], the glycation of proteins [15] and the activation of the polyol metabolism [16]. These changes accelerate the generation of ROS, and result in an increase in the oxidative modification of lipids, DNA and proteins in various tissues. Oxidative stress may play an important role in the development of complications in diabetes.

The "Akita mouse" is a nonobese model of early-onset non-insulin dependent diabetes mellitus (NIDDM), and characterized by the early age onset autosomal dominant mode of inheritance. In the pancreas of Akita mice, the number of active β-cells decreased [17, 18, 19]. Furthermore, the mutation of the insulin2 gene is clearly demonstrated in the Akita mouse, and recognized as a major factor of hyperglycemia of this animal. Hydronephrosis is also commonly observed in the kidneys of Akita mice [17]. There are some reports that the levels of 8-OHdG in the urine and mononuclear cells were increased in patients with type 2 diabetes [20, 21]. Ihara et al. have reported that the levels
of the 8-OHdG and HNE-modified proteins are significantly increased in the pancreatic β-cells of diabetic GK rats [22]. However, there has been no prior studies on whether Akita mice encounter oxidative stress which results in the oxidative modification of biomolecules caused by hyperglycemia.

In this study, we mainly focused on Akita mice, a model for type 2 diabetes, and investigated the relationship between hyperglycemia and

oxidative stress during early aging. We show here for the first time that the oxidative stress has already occured in the kidneys for young Akita mice, and it can be estimated by the detection of the lipid hydroperoxide-modified protein and protein cross-linking using the anti-HEL and anti-DT antibody, and they may be possible markers for NIDDM.

2. Materials and methods

2.1 Experimental design and tissue preparation

Akita mice (male, 5-week-old) and C57BL/6CrSlc (male, 5-week-old) mice (Japan SLC, Inc., Hamamatsu, Japan) were used and housed in plastic cages at 23±3 °C with a 12-hour light cycle. Mice were maintained in accordance with the Guidelines for Animal Experimentation of Nagoya University. These mice allowed free access to tap water and stock chow (CE-2, Japan CLEA, Tokyo, Japan) for 8 weeks. After 8 weeks of feeding, the mice were killed under anesthesia with diethyl ether. The serum was obtained from the collected blood by centrifugation at 10,000 rpm for 3 min at 4 °C. The liver and kidneys were rapidly excised, and kept at -80 °C until use.

2.2 Determination of blood glucose concentration

Blood samples were obtained from the tail veins of mice at 6, 8, 10 weeks. The serum glucose concentration was enzymatically determined with a commercial assay kit (Glucose B-Test Wako, Wako Pure Chemical Industries, Ltd., Osaka, Japan).

2.3 Antibody

2.3.1 Anti-HEL antibody and anti-DT antibody

The monoclonal antibody specific to HEL was prepared as follows. Hexanonyl KLH and its analog, hexanonyl BSA, were prepared from hexanoic

acid and proteins with EDC and sulfo-NHS as already described [5]. BALB/c mice were intraperitoneally primed with the modified KLH emulsified in Freund's complete adjuvant. Mice were boosted twice at a 2-week interval. Three days after the intraperitoneal final injection of the antigen without adjuvant, the animals were sacrificed, and the P8U1 myeloma cell line and the spleens of the immunized mice were fused using polyethylene glycol. The production and selection of the hybridomas were carried out by the method of Kohler and Milstein. The supernatants of the culture were used for the enzyme-linked immunosorbent assay (ELISA). The positive clones were selected using hexanonyl BSA as the antigen for ELISA. The obtained hybridomas obtained were intraperitoneally injected into the BALB/c mice, which had been injected with pristane (2,6,10,14-tetramethylpentadecane) one week before, and about 7-10 days later, the ascites were collected and partially purified by ammmonium sulfate fractionation. The anti-HEL antibody recognizes the HEL moiety [Kato, Y., Miyake Y., Yamamoto, K., Shimomura, Y., Ochi, H., Mori, Y., and Osawa, T.; manuscript in preparation] compared to the polyclonal anti-HEL antibody [5].

The monoclonal antibody specific for protein DT was prepared as follows. Briefly, the dimer of 3-(p-hydroxyphenyl)propionic acid(HP) was prepared by incubation of HP with HRP in the presence of H_2O_2. The product was purified and identified as a dimer of 3-(p-hydroxyphenyl) propionic acid, named di-HP, by mass spectrometry. The obtained dimer(di-HP) was conjugated with KLH using EDC and N-hydroxysucciniimide. The immunization of di-HP-KLH into mice and the isolation of positive clones were done as described above. To obtain the monoclonal antibody to the di-hydroxyphenyl moiety, the clones were selected using dityrosine-conjugated BSA (DT-G-BSA) as the antigen for ELISA. The ascites was obtained as described above. Partially purified antibodies were prepared using ammonium sulfate fractionation and the used in

the experiments. The obtained antibody reacts with dityrosine itself and H_2O_2/Cu-oxidized proteins [Kato,Y., Kitamoto, N., Kawai,Y., Wu, X. H., Nomura, H., Naito, M. and Osawa, T.; manuscript in preparation]. The detailed characterization will be shown elsewhere in the future.

2.4 Enzyme-linked Immunosorbent Assay (ELISA)

Each tissue sample (liver and kidney) was homogenized in 9 volumes of 100 mM phosphate buffer (pH 7.0) using a Potter-Elvehejm homogenizer. The homogenate was centrifuged at 10,000 rpm for 10 min at 4 °C and used as samples for the ELISA. Fifty μl of a sample (0.01 mg proteins / ml) was pipetted onto each well and kept at 4 °C overnight. After washing three times with phosphate-buffered saline containing 0.05% Tween 20 (TPBS) and water, the plate was blocked by 1% Block ACE (Dainihon Seiyaku, Osaka, Japan) for 1 h at 37 °C. After washing, 100 μl of monoclonal anti-HEL antibody (diluted 1:4000) or the monoclonal anti-DT antibody (diluted 1: 50000) was added to each well and soaked for 2 h at 37 °C. After washing with TPBS and water, 100 μl of the peroxidase-labeled anti-mouse IgG antibody (Organon Teknika Co.) was reacted for 1 h at 37 °C. After washing, 100 μl of *o*-phenylenediamine solution (*o*-phenylenediamine 5 mg/ 30% H_2O_2 10 μl / 50 mM citrate-phosphate buffer (pH5.5) 10 ml) was added to each well. The plate was periodically shaken at room temperature for 20 min, then the reaction was terminated by 2 N sulfuric acid, and the absorbance at 490nm was measured with a multiplate reader.

2.5 Immunohistochemistry

A part of the kidneys were fixed in 4% paraformaldehyde. The kidney specimens were embedded in paraffin and sectioned. The sections were deparaffinized with xylene and ethanol. For the detection of the

HEL-modified protein and DT, the avidin-biotin complex (ABC) method was performed using a commercial Kit (VECTASTAIN Elite ABC KIT,. Vector Laboratories, Burlingame, CA, U.S.A.). After deparaffinization, endogeous peroxidase was quenched with 3% H_2O_2 in phosphate buffered saline (PBS) containing 10 % methanol for 5 min. The sections were permeabilized with 0.1% Triton X-100 in PBS for 20 min. The sections were incubated with normal horse serum (VECTASTAIN) for 1 h at room temperature. The sections were then incubated for 4 h at room temperature with anti the HEL monoclonal antibody diluted 1:500 in PBS or with monoclonal anti-DT antibody diluted 1:5000 in PBS. They were then incubated with biotinylated Universal antibody (VECTASTAIN) for 1 h at room temperature as the secondary antibody. The sections were then incubated with ABC reagent (VECTERSTAIN) for 30min, and positive reactions were visualized by incubation with the commercial peroxidase substrate (SIGMA FIRST DAB, SIGMA) solution containing diaminobenzidine, urea, and H_2O_2. Nuclei were counterstained with methyl green. DT staining followed a similar procedure, except the anti-DT antibody was diluted 1:5000 in PBS.

2.6 Specificity of immunostaining

To confirm the specificity of the antibody, absorption tests were performed by adding free antigens during the incubation of each primary antibody on glass slides. Hexanonyl-BSA [5] (1mg/ml) was incubated with the anti-HEL antibody at 4 °C overnight, on the other hand, DT-G-BSA [6] (1mg/ml) was reacted with anti-DT antibody at 4 °C overnight. Each reaction mixtures was centrifuged and the supernatant was used for examination of specificity of immunostaining. After staining by the same procedures described above using the antigen-antibody complex, the stained sections were compared.

2.7 Statistical analysis

The results are expressed as means ± SD. Data are compared using Student's *t*-test. $P<0.05$ was considered significant.

3. Results and discussion

3.1 Hyperglycemia of Akita mice

Fig.1 shows the growth of the Akita and control mice during the experiment. No significant difference was observed in the growth between the Akita and control mice. This result shows that Akita mice are nonobese models for type 2 diabetes. Fig. 2 shows the serum glucose concentrations in the Akita and control mice. During the course of the experiment, the serum glucose concentration in the Akita mice was above 400 mg/dl, while in the control mice, it was less lower than 170 mg/dl.

3.2 Increases in HEL in kidneys of Akita mice

Kato et al. previously reported that HEL was clearly detected in the atherosclerotic lesions of human using the antibody specific to HEL [5]. This result suggests that HEL is a useful biomarker for the early stage of oxidative stress in vivo. To investigate the presence of the lipid peroxide-derived protein covalent modification on type2 diabetes, we evaluated the HEL levels in the liver and kidneys of Akita mice by ELISA using the anti-HEL antibody. No significant difference was observed in the HEL levels between the Akita and control mice at 13weeks of age (Fig. 3A). In contrast, the HEL level in the kidneys of Akita mice was significantly higher than that of the control strain (Fig. 3B). These results suggest that hyperglycemia induces the production of lipid peroxide-derived protein and covalent modification lipid hydroperoxide-modified protein in the kidneys of Akita mice at an early age.

Furthermore, to confirm the increase and to examine the localization of HEL in the kidneys of the Akita mice, we carried out an immunohistochemical analysis of a kidney section using the anti-HEL antibody. Immunopositive staining at the distal covoluted tubule was clearly observed in the kidneys of the Akita mice (Fig. 4A). On the other hand, only weak staining was observed in the kidneys from the control mice (Fig. 3B). Although the data are not shown, using the antigen-antibody complex showed only weak staining.

It is known that lipid peroxidation products can modify biological materials. Among the products, the reactivities of malondialdehyde (MDA) and HNE have been investigated in detail, and it has been shown that MDA or HNE-modified proteins and thiobarbituric acid reactive substances (TBARS) [23] were increased in diabetes [24]. However, in this study, TBARS and HNE-modified proteins were not significantly increased in the kidneys of Akita mice compared to that of the control mice (data not shown). It was recently reported that HNE levels are significantly increased in the pancreatic β-cells in GK rats [22]. These results suggest that advanced lipid peroxidation end products were not accumulated in the kidney at the age of 13 weeks in Akita mice, and HEL is a useful marker for the initial stage of oxidative stress of type 2 diabetes, without the accumulation of advanced lipid peroxidation end products in the kidneys.

3.3 Increase of DT in kidneys of Akita mice

DT is formed by active oxygen species [8, 9], enzymatic reactions [10,11], UV irradiation [12] and lipid peroxidation [13]. Recently, Kato et al. developed an anti-DT antibody and showed that DT was detected in the lipofuscin of the aged human brain using the antibody [6]. Therefore, DT could become a useful marker for protein cross-linking [6]. In order to detect the protein cross-linking in type 2 diabetes, we evaluated the DT level in the liver

and kidneys of Akita mice by ELISA using the anti-DT antibody. In the liver, no significant difference was observed in the DT level between the Akita and control mice (Fig. 5A). On the other hand, the DT level in the kidneys of Akita mice was significantly higher than that of the control mice (Fig. 5B). These results suggest that hyperglycemia increases the protein oxidation in the kidneys of Akita mice at an early age. Fig.6A shows the immunonhistochemical analysis of the kidneys in Akita and control mice using the anti-DT antibody. Immunopositive staining at the proximal covoluted tubule was clearly observed in the kidneys of the Akita mice (Fig.6A), but not at the diatal covoluted tubule. These results indicate that the localization of HEL and DT are different in the kidneys of the Akita mice with hyperglycemia. On the other hand, in the kidneys of the control mice, only weak staining was observed (Fig.6B). In the kidneys of the control strain, only weak staining was observed. No staining was shown in the absorption test using the DT-G-BSA and the anti-DT antibody (data not shown). After 30 weeks, hydronephrosis is observed in the kidneys of the male Akita mice[14]. The accumulation of lipid peroxide-derived protein covalent modification or DT in the kidneys of Akita mice may become one of the causes of hydroneprosis.

In conclusion, we showed that hyperglycemia in Akita mice induces an increase in HEL and DT in the kidneys at an early stage of age without elevation of the HNE-modified protein and TBARS. These results suggest that oxidative stress occurs in the kidneys at the early age for Akita mice and it can be estimated by the detection of the novel biomarkers. OLETF rats, GK rats and Akita mice are typical models for type 2 diabetes, but they have long time to generate the diabetic complications. The detection of HEL and DT could be expected to detect the diabetic complications at an early age which can be then easy to control in diabetic patients.

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Figure legends

Fig. 1 Body weight growth as a function of age. Means and SD of body weight for Akita mice and control mice are shown. Akita mice (●) and control mice (○) are represented.

Fig.2 Blood glucose levels in Akita mice and control mice as a function of age. Means and SD of blood glucose for Akita mice and control mice are shown. Akita mice (●) and control mice (○) are represented.

Fig.3 HEL levels in the kidney (A) and the liver (B) of Akita and control mice determined by ELISA. Values are means ± SD for 4 mice. *Significantly different ($p<0.01$) from the control value.

Fig.4 Immunohistochemical detection of HEL in the kidney of Akita mouse. Strong staining was observed in the distal covoluted tubule of Akita mouse (A). Negligible antibody reaction was observed in the kidney of control mouse (B). Methylgreen nuclear counterstaining was used; original magnification. ×200.

Fig.5 DT levels in the kidney (A) and the liver (B) of Akita and control mice

determined by ELISA. Values are means±SD for 4 mice. *Significantly different ($p<0.05$) from the control value.

Fig.6 Immunohistochemical detection of DT in the kidney of Akita mouse. Positive staining was observed in Akita mouse (A). Weak staining was observed in the proximal covoluted tuble of control mouse (B). Methylgreen nuclear counterstaining was used; original magnification. ×200.

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Fig. 1

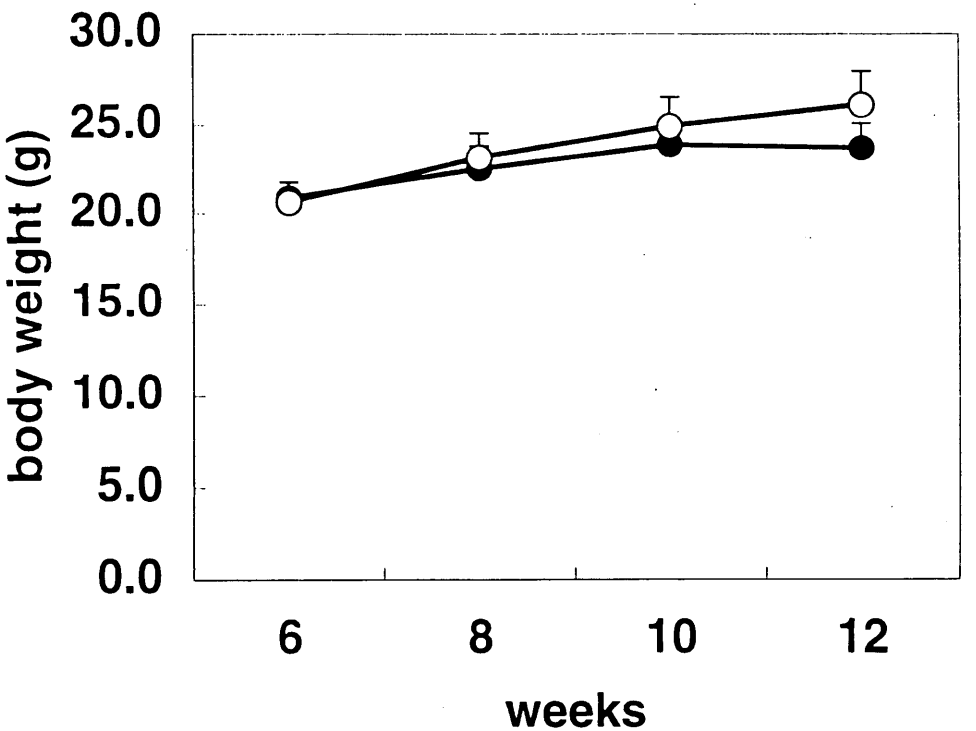
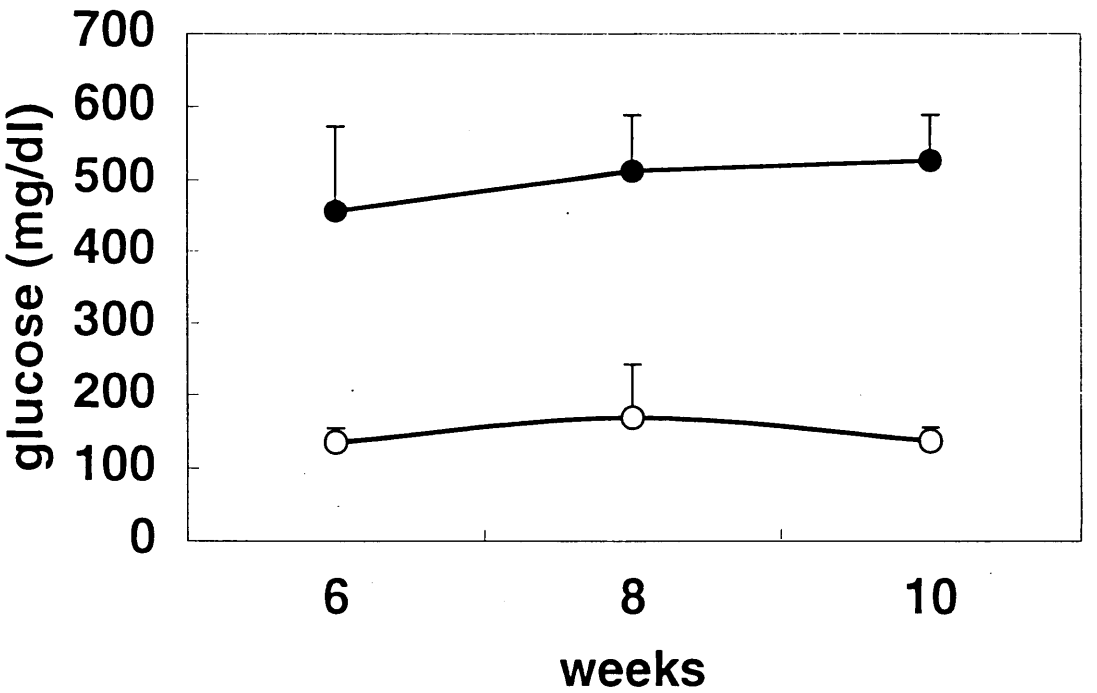
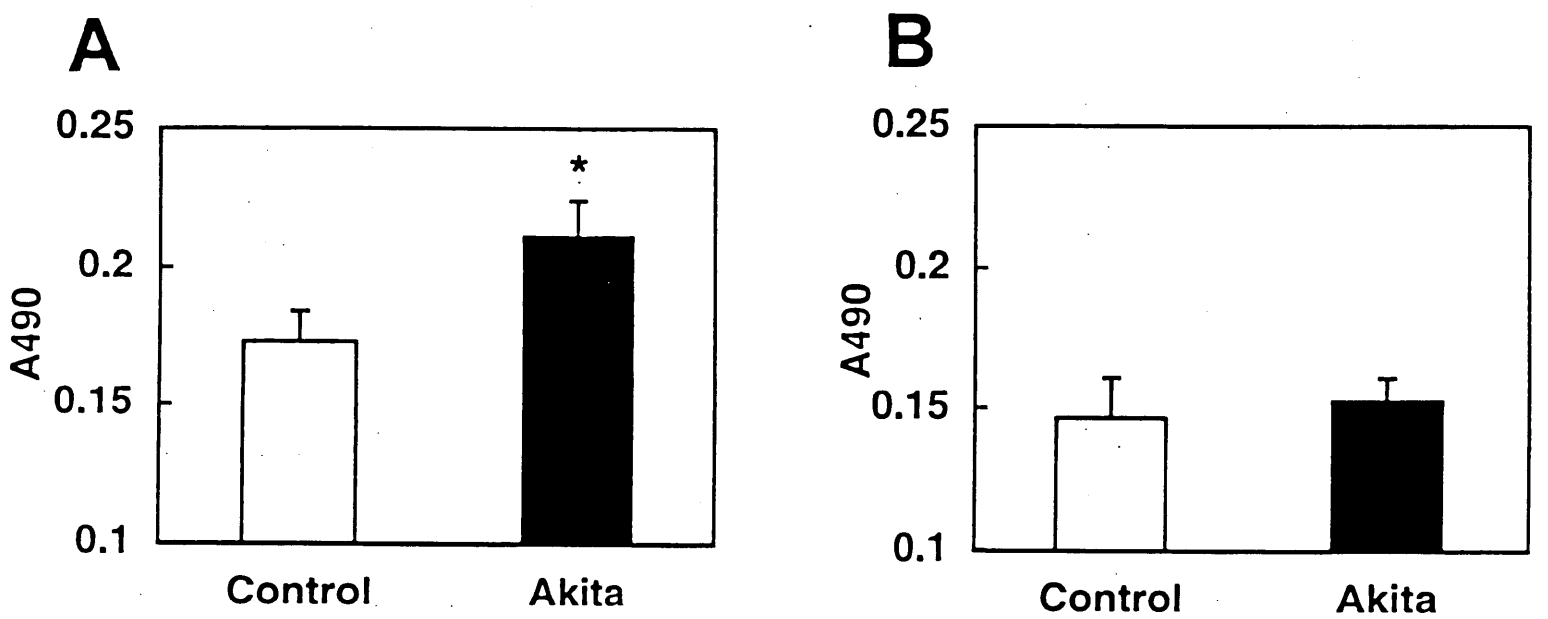


Fig. 2

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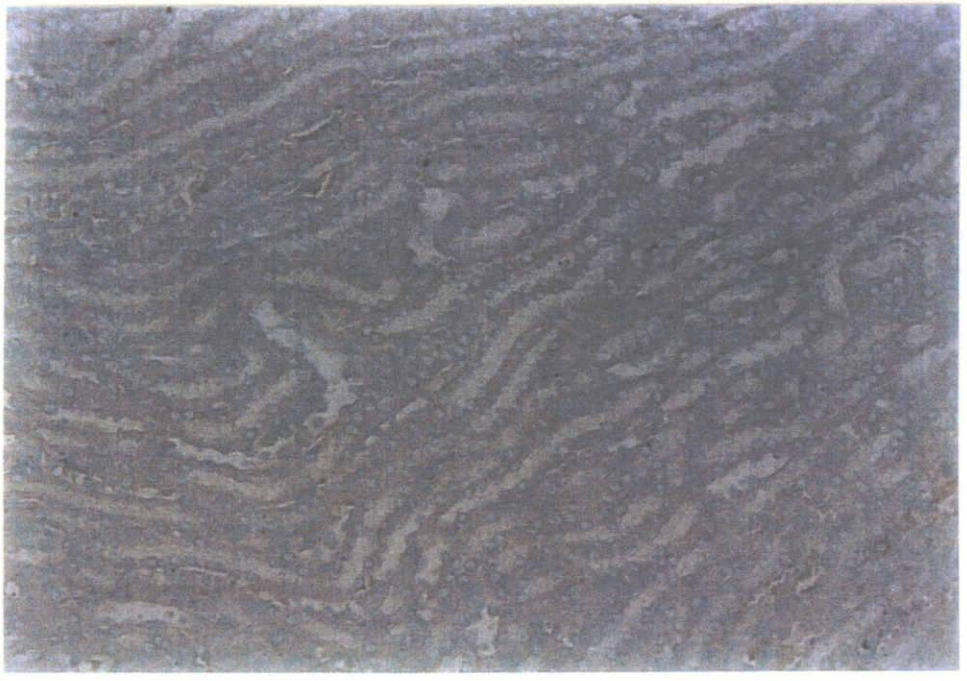

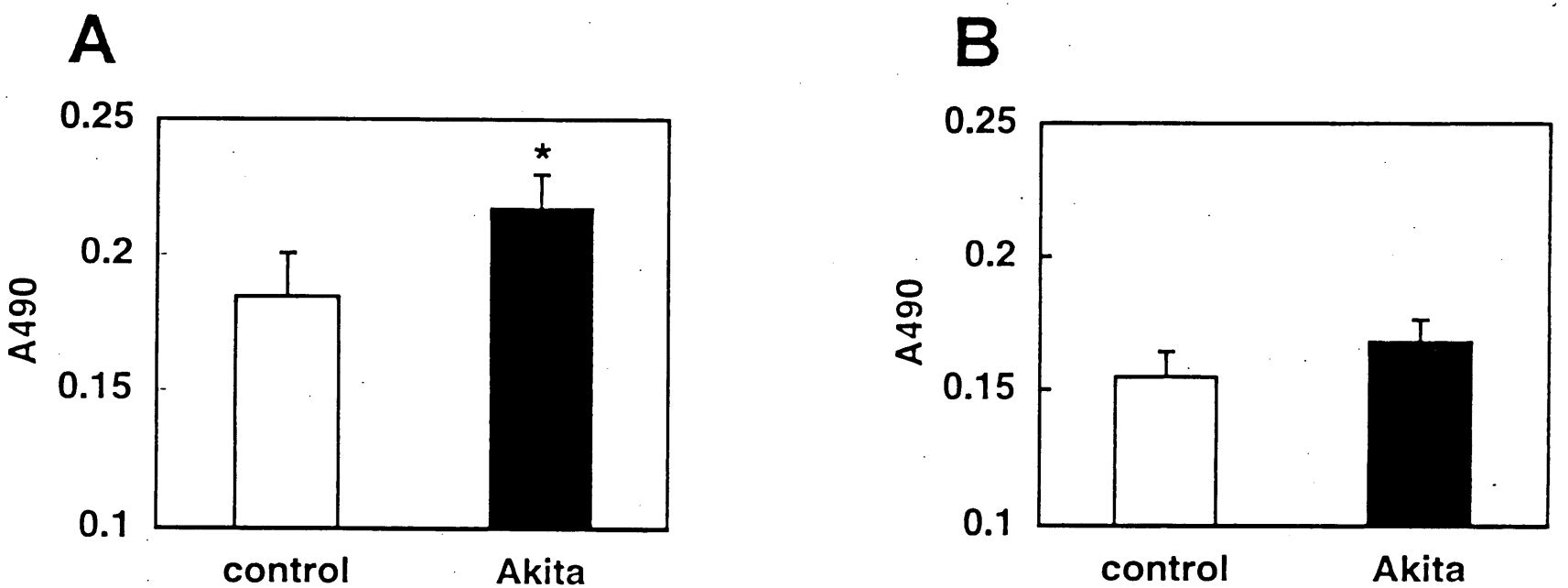
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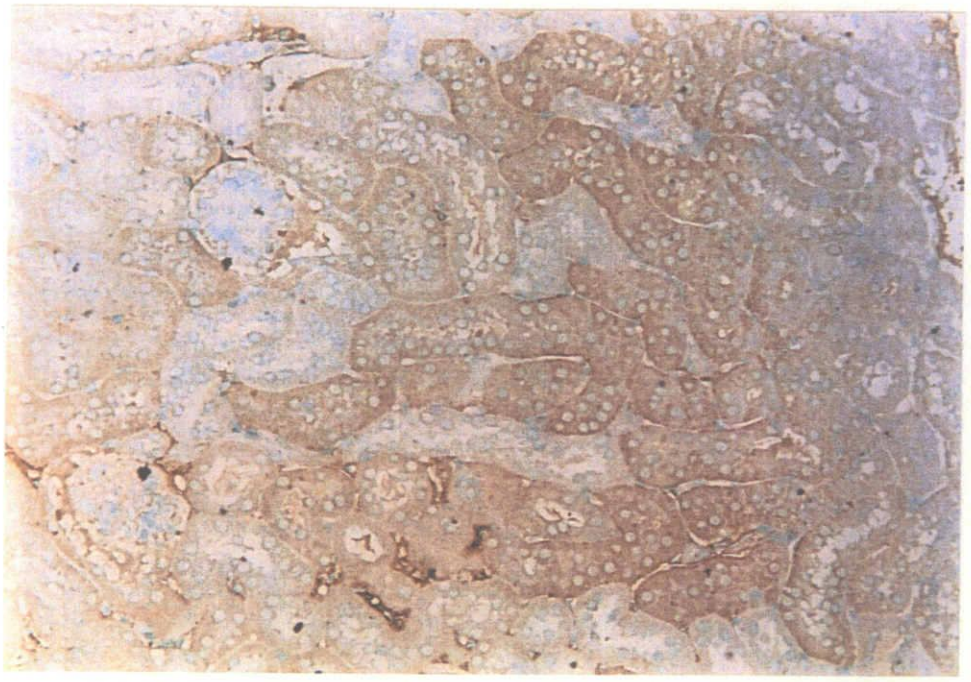

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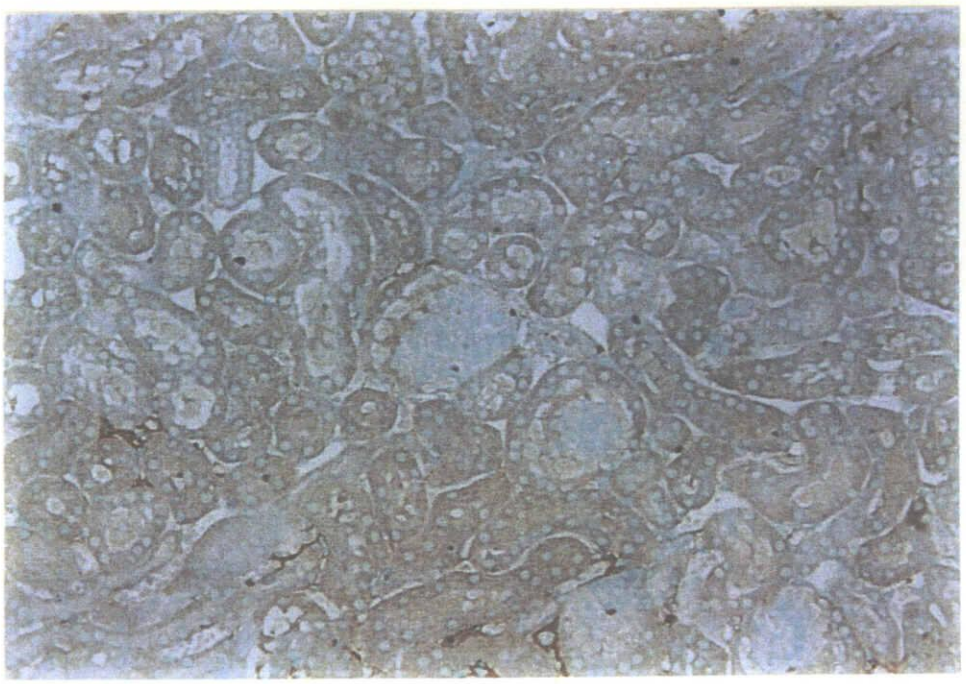

３．クルクミン、テトラヒドロクルクミンによる糖尿病合併症予防効果

われわれの研究グループは、長年、ウコン（ターメリック）に注目して研究を進めてきた。沖縄では、「ウコン茶」とか「ウッチン茶」とよばれ伝統的に愛用されてきたが、世界的には「ターメリック」としてインド料理をはじめとする香辛料としての役割のほうがよく知られている。われわれの研究グループが「ウコン（ターメリック）」に注目したのは、主成分である黄色色素「クルクミン」の生体内代謝物である「テトラヒドロクルクミン」の持つ強力な抗酸化性であった。われわれの進めている「酸化ストレス予防食品開発」の基盤的研究には、「生研機構」からのサポートもあり、また、世界的にも大きな注目をあつめつつある。このように抗酸化食品が注目された背景には、「活性酸素・フリーラジカルは「諸刃の刃」であり、われわれの体は一般に酸化―抗酸化のバランスがとれているのであるが、何らかの原因で過酸化側の方にバランスが崩れたときに「酸化ストレス」が生じ、この「酸化ストレス」による酸化傷害を予防することで、「がん」をはじめとする生活習慣病を予防できるのではないか、との熱い期待がある。

一般に、われわれが日常口にする食品は、量的にはバラツキがあるもののほとんどすべての食品中に何らかの抗酸化成分が含まれており、しかも、その多くは植物由来である。例えば、油糧種子や穀類、植物のリーフワックスやハーブなど多種多様な植物素材があげられているが、特に注目されるのは、強い光に曝され、また厳しい酸素ストレスのもとで生育している植物に含まれている抗酸化成分である。すなわち、高温に晒され、しかも、強力な紫外線のもとでたくましく生育する沖縄に独特の植物には、自分自身を守り、また、生命を次世代に受け継ぐために、植物体内に抗酸化物質を持っているのではないか、との考えである。しかも、このような植物中の抗酸化成分は、植物自身の酸化的障害からの保護に重要な役割を果たすと共に、そのような抗酸化成分を我々人間が摂取することにより老年病を予防し、老化制御への道を探ることができるのではないかと考えたわけである。

沖縄で一般的に飲まれている「ウッチン（ウコン）茶」の素材は「アキウコン」であり、また、「ターメリック」としてインド料理には不可欠の香辛料でもある。沖縄には多くの注目すべき伝統的な食素材が存在しているが、沖縄で最も広く広まり、また、この「ウコン」の持つ新しい機能性、特に、「がん予防効果」には世界中の研究者が熱い視線を向けており、例えば、京都府立医大の西野教授は、「ウコン」の黄色色素「クルクミン」こそ、新しいがん予防の戦略物質である、と強調されている。沖縄の長寿食として数多くの食品素材があげられるが、私が長年研究に携わってきている「ウコン」は、沖縄伝統の食素材のなかで最も研究が進んでいるもので、また、世界的にみても、がんをはじめとする疾病の予防の最有力の「フードファクター」として期待され、また、個人的にも、沖縄滞在を通じて「ウッチン茶」を飲む習慣が沖縄の長寿の原因の一つと言われていることを身を持って体験したものとしても、本稿では、特に、この「アキウコン」の主要な黄色色素である「クルクミノイド」のもつ「がん予防効果」に焦点をあてて最近の話題を紹介して行きたい。

亜熱帯に育った「アキウコン」は零下の世界では生きて行けないので、日本ではほとんど栽培されず伝統的に沖縄のみで生産されてきた。江戸中期時代には、「鬱金（ウコン）」と呼ばれた「アキウコン」（*Curcuma longa* L.）はかつてはシャムから多くきたが今は琉球から江戸へ多く運ばれ、木綿や紙を染色する目的で多く用いられた、と記載されている。「鬱金」は、沖縄では単に染色用だけでなく、当時特効薬のなかった結核、肋膜、喘息といった病気に効果を示すものとして、珍重され、さらに、ウコン茶（ウッチン茶）や発酵ウコン茶など、スパイスとしてよりもお茶の素材として伝統的に用いられてきたのである。しかしながら、世界的に見ると「アキウコン」の最大の生産国はインドであり、ほとんどが香辛料「ターメリック」として利用されている。毎年１０万トン以上が生産されるが、９５％は国内で消費され、インド以外では、中国南部の広東省でも生産され、ごく一部であるが台湾でも栽培され日本へ輸出されている。

この「アキウコン」の名前の由来は、秋（８－１１月）に花を咲かせることに由来するが、ターメリックの近縁な植物として「ハルウコン」が知られている。「ハルウコン」とい

うのは、同じショウガ科クルクマ属のCurcuma aromatica Salisb.で、インド原産であると共に沖縄でも自生している。この「ハルウコン」は、名前の通り、４ー６月ごろ、葉のでる前か同時に開花し、主として生薬として用いられ、余り食用には用いられない。ハルウコンは、別名、キョウオウと呼ばれ、見た目には「アキウコン」によく似た植物体で花も似ているので区別はつきにくいが、「アキウコン」の葉の裏側がつるつるしているのに比べて「ハルウコン」の葉の裏には毛があり、ざらざらした感触がある。しかし、最も異なるのは、ハルウコンの根茎には黄色色素の含量が少ないために薄い黄色である。また、同じ近縁の植物として、「シロウコン」とか「ムラサキウコン」と一般的に呼ばれる日本名が「ガジュツ」(Curcuma zedoaria Rosc.)も、形態は「アキウコン」や「ハルウコン」と似ており、「ハルウコン」と同じように春に紫がかった白色をした花を咲かせる。「ガジュツ」の根茎は紫がかった白色で、インド原産であるがスリランカやインドネシアで栽培され、ベンガルや南インドではカレーの賦香料としても用いられ、また、根部のデンプンを食糧に用いられる場合もあるが、強い苦みを持ち、主として薬用に用いられている。

このように、近縁の「ウコン」にはいくつか種類があるが、どうもその名称には若干の混乱も生じてきている。例えば、生薬では大先輩の中国では、一般に「鬱金」といわれているのは「ハルウコン」(*Curcuma aromatica* Salisb.)であり、逆に薑黄（キョウオウ）と称されているのが *Curcuma longa* L.である。しかしながら、何故、このように生薬の薬物名と植物名が中国と日本とは全く逆になったのか、その理由は不明である。

「アキウコン」の主成分は「クルクミノイド」と呼ばれる３種類のクルクミン類縁体の混合物である。この鮮やかな黄金の色が太陽の色として「鬱金」の最も重要な役割であった。勿論、クルクミンが主成分であり、色素成分の８０％以上を占めている。香辛料としての「ターメリック」の品質の評価には、このクルクミンの含量が重要な指標となる。「クルクミン」の利用として特によく知られているのは、沢庵漬けの黄色である。沢庵の黄色は、塩分と微生物による自然の発色は、どうしても黄色になり難く、黄色を強めるためには塩分を加えるという問題点があった。低塩化が叫ばれる今、沢庵漬にクルクミンは必須となっている。その外にも、ウインナーソーセージの羊腸の外側を染める目的で用いられたり、クリやリンゴなどのシロップ漬けにも利用されている。 勿論、ターメリックは他の数多くのスパイスと混合され、カレーパウダーとして用いられる場合が最も多く、インドでは、ターメリックをほとんど毎日のように料理に用いられ、特に芳香性や辛味効果を期待するために、調理の前にターメリックをはじめ１０数種のスパイスをブレンドしてカレーパウダーを作る、ということはそれぞれの各家庭に伝統的な味が引き継がれている。このように、沖縄では「ウコン茶」や「ウッチン茶」としての利用が圧倒的に多い「ウコン」であるが、世界的には圧倒的に、香辛料、「ターメリック」として用いられている。私自身、この「ウコン」の機能性に関する研究に携わって１０年弱になるが、最近の「ウコン」に対するマスコミの取り上げ方は、時には異常とも思えるほどである。

最近、私の長年の友人である台湾大学医学部林(J-K.Lin)教授が中心になって、日本を含めた東南アジアで多い「肝がん」の原因であるウイルス性肝障害の患者を対象に、がん予防物質として「クルクミン」の投与研究が始められた。このような人を対象にして、ある食品成分を投与する研究を「介入研究」をよばれ、一般に５年以上かかり莫大な費用のかかる大規模な研究である。わたしたちは、「クルクミン」が本当にヒトのレベルで肝炎ウイルス由来の「肝硬変」、最終的には「肝臓がん」を予防できるかどうか、結果が期待されている。１９９９年１２月での第２回 ICoFF、すなわち、「国際フードファクター学会」にJ-K.Lin教授の招待講演が予定されており、中間報告ではあるが、研究の詳細を知ることができるものと楽しみにしている。最近の「ウコン」の肝機能に対する研究成果としては、東京薬科大学の糸川秀治教授のグループを中心に、肝炎や肝障害に対する有効性を示した動物試験の結果や、胃酸や胆汁の分泌を著しく促進し、胃腸の働きを高める効果などのデータ示されている。このような「ウコン」の持つ生理機能の中心は、黄色色素である「クルクミン」であろうと考えられているが、「ウコン」、すなわち「ターメリック」には多く

の成分が存在しているので、これらの作用のことも頭に入れておくべきであろう。「肝臓がん」は日本でも増加し続けており、いずれは、がん死亡率の第一位を占めるようになるのではないか、と警告されている。その原因は、肝硬変を経て肝臓がんに至る肝障害であるが、世界的に見てみると、アジア諸国は欧米に比べてウイルス性肝障害が圧倒的に多く、欧米では逆にアルコール性の肝障害が圧倒的に多い。ウイルス性肝障害の原因となる肝炎ウイルスは、A型、B型、C型、D型、E型の５種類が存在している。A型肝炎は経口感染で、日本では、上下水道の完備と共に減少しているが、衛生状態の良くない東南アジアやインド、中近東やアフリカ、南アメリカなどで生水や氷、生カキなどから感染する場合が多い。しかし、問題となるのは、B型肝炎、C型肝炎である。これらは、血液や唾液など、体液を介して感染する場合が多く、しかも慢性化し、最終的には肝硬変を経て肝臓がんに至る、という訳である。日本での肝硬変の患者の数は約１１万人といわれ、その多くは、B型やC型肝炎ウイルスによるものであるが、一方、欧米では肝硬変の大部分がアルコール性肝障害によるものである。アルコールを飲み続けると、まず、アルコール性脂肪肝になり、さらに飲酒量が増えるとアルコール性肝炎となり、最終的に肝硬変にいたってしまう。沖縄で有名な「エイサー」という、本土でのお盆にあたるお祭りがある。「エイサー」では、泡盛を浴びるほど飲み続け、３日間、踊り続けるのである。この時に重要なのが「鬱金」である。二日酔いの朝に、「鬱金」を摺って水と味噌を加え、それを飲み干すことが最善の策であるといわれている。

実際、沖縄のマーケットを歩いてみると、至る所で「鬱金」が売られ、また、「ウッチン茶」が店先に並べられている。特に、最近注目を集めているのが「発酵ウコン茶」である。この「発酵ウコン茶」は、琉球大学農学部本郷富士弥教授と琉球バイオリソース（株）により商品化され、発酵により「ウコン」の持つ特有の土臭さと苦みがやわらいで、飲みやすいお茶となっている。実は、「発酵」という伝統的な食品加工法には、われわれの研究グループ、特に、椙山女学園大学の江崎秀男助教授を中心に興味ある研究結果を発表してきている。日本の伝統的な発酵大豆食品である納豆や味噌、また、インドネシアの伝統的な発酵大豆食品のテンペなどは、いずれも、原料である蒸し大豆に比べて強力な抗酸化性を有するようになることを明らかにしてきている。納豆は、枯草菌、味噌は麹菌、また、テンペはクモノスカビ、と用いる微生物が異なっているので、それぞれ、特徴のある味や香りを持ち、また、抗酸化性分も異なっていることを発表してきた。「発酵ウコン」というのは、乳酸菌を利用している点で特徴があり、「琉球バイオリソース」と私たちの研究室との共同研究で、やはり、強力な抗酸化性を示すようになることを見出している。実際に、沖縄の勝山病院では、８０歳以上の老人に、この発酵ウコンの粉末を毎日２ｇ、１２週間投与することにより、ＤＮＡ酸化傷害マーカーとしてわれわれが最近開発したモノクローナル抗体を利用したELISAによる測定の結果、尿中に排泄される8-OH-dGの量が減少することを明らかにし、酸化ストレスに対する予防効果が期待された。「酸化ストレス」が、成人病や生活習慣病と呼ばれる疾病の大きな原因の一つであり、この「発酵ウコン茶」の新しい機能性がこれから次々と明らかにされてくるものと、期待されている。

「鬱金」は漢方でも止血剤や健胃剤としては用いられ、また、インドやマレーシア、インドネシアなどで、特に、女性はターメリックを皮膚に塗る習慣があることは、既に紹介した通りである。「鬱金」には抗菌作用や抗炎症作用があることは、古くから知られており、単に化粧として塗られるだけでなく、経験的にこのような効能を利用し、紫外線による傷害や皮膚感染などを予防したものであろう。この「鬱金」の研究が世界的に進められたのは「鬱金」の主要な黄色色素である「クルクミノイド」であり、特に、80%以上を占める「クルクミン」のがん予防効果である。がんの発生のメカニズムとして一般的には「発がん多段階説」が受け入れられており、初期段階である「イニシエーション」、促進過程の「プロモーション」、悪性化の段階の「プログレッション」という少なくとも３段階が存在する。最近、アメリカと日本で、この「クルクミン」に強力な発がんプロモーションの抑制作用が見出され大きな注目が集められた。発がん研究で最も良く用いられる動物モデルが「皮

膚がん」のモデルである。皮膚がんの動物実験は、マウスを用いて皮膚に発がんを起こさない量の発がん剤、7,12-dimethyl benz[a]anthracene(DMBA)を塗布しておく。そこに、発がん促進剤、12-O-Tetradecanoyl phorbol-13-acetate (TPA) を塗ると皮膚がんを起こす、という実験である。TPA と共に「クルクミン」を塗ることにより、がんの促進化を抑えた、というデータが、アメリカ、ニュージャージー州立のラトガース大学がん研究所の Conney らが最初に明らかにした。これらの皮膚がんに対するクルクミン誘導体の抑制効果は「クルクミン」が最も強力であった。その抑制機構については、京都大学大東教授のグループが研究を進め、Nakamura らにより発がん促進過程で生成されたフリーラジカルの捕捉能との間に大きな相関性があることを報告している。

では、「クルクミン」の経口摂取での「がん予防効果」はどうであろうか？ Huang らは、一般的に入手できる「クルクミン混合物」(77%:Curcumin, 17%:demethoxycurcumin, 3%:bisdemethoxycurcumin) を化学発がんモデルマウスに投与して、前胃がん、十二指腸がん、大腸がんに対しての抑制効果を報告している。Benzo(a)pyrene による前胃がんの初期過程では 51-53%抑制し、促進過程に投与しても 47-67%の抑制効果を見出し、N-ethyl-N'-nitro- N-nitrosoguanidine で誘導された十二指腸がんでは 47-77%促進過程を抑制し、azoxymethane 誘導の大腸がんモデルでは、2%のクルクミン投与で、66%初期過程を抑制し 25%促進過程を抑制しており、「クルクミン」は皮膚塗布だけでなく経口投与でも「がん予防効果」を示すことが期待できた。さらに、アメリカ健康財団の Reddy らは、azoxymethane 誘導の大腸がんモデルでさらに詳細に大腸がん抑制メカニズムの検討を行い 0.2%の投与で azoxymethane 誘導の大腸がんモデルの促進過程を 57%以上抑制し、phospholipaseA2 を抑制すると共にシクロオキシゲナーゼ抑制によるアラキドン酸カスケード関与の抗炎症作用との関連を示唆している。

さらに、最近、Inano は、われわれとの共同研究で、γ―線照射による乳腺腫瘍の促進過程を有効に抑制したことを報告している。Diethylstilbestrol をプロモーターに用いたラットでの乳腺腫瘍に対して 1%の経口投与でコントロールに較べて 28%の腫瘍の生成頻度まで減少した。その抑制機構についての解析を行い、発がん促進過程における「酸化ストレス」を抑制していることを明らかにした。また、日本では、京都府立医科大学の西野輔翼教授は、「β―カロテンのがん予防効果に疑問がでている今、世界的に最も注目されるがクルクミンである」と述べているので、今後、クルクミンの研究は、益々注目されてくるであろう。

「クルクミン」は、皮膚に塗る場合と食べる場合とで同じ効能が考えられるのであろうか。ここで登場するのが「テトラヒドロクルクミン」という物質である。最近、この「クルクミン」も経口で摂取すると腸管の部分で「テトラヒドロクルクミン」という強力な抗酸化物質に変わることを明らかにすることができた。「テトラヒドロクルクミン」というのは、腸の細胞で吸収されるときに「クルクミン」が変化してできる物質で、私たちが「クルクミン」を食べると吸収される時に「テトラヒドロクルクミン」に変換され、体の中で実際に効果を示すのはこの「テトラヒドロクルクミン」である、という訳である。

われわれが、最初に「テトラヒドロクルクミン」を生み出したのは、ユーカリ葉のリーフワックス中の強力な抗酸化物質として存在を明らかにすることができたβ―ジケトンタイプの抗酸化物質の研究であった。すなわち、「クルクミン」自身は黄色色素としての利用も考えられ、実際、日本ではタクワン漬けに多く用いられているが、食品用の抗酸化剤として広く利用するためにはこの黄色は逆に汎用性という面ではマイナスではないかと考え、この「クルクミン」を接触還元することでβ―ジケトン構造を導入することを考えた。実際に、主成分である「クルクミン」と共に微量にしか存在しない２種類の「クルクミン類縁体」を接触還元により３種類のテトラヒドロ体を得ることができ、抗酸化性を測定したところ、いずれも抗酸化性が増強され、特に、「テトラヒドロクルクミン」に最も強い抗酸化性が見出された。そこで、まず最初に、この「テトラヒドロクルクミン」の食品用抗酸化剤としての応用の可能性を探っていたところ、興味ある結果を得ることができた。すな

わち、「クルクミン」を動物に投与したところ、血中には「クルクミン」は存在せず、「テトラヒドロクルクミン」に変換される、という結果であった。実際に、培養細胞を用いても、この「クルクミン」から「テトラヒドロクルクミン」の変換を観測することができ、この結果は、「クルクミン」を摂取したときの主要な代謝物は「テトラヒドロクルクミン」であることを示している。

そこで、まず、国立がんセンターのTsudaらのグループとの共同研究により大腸がんの予防効果の検討を行うことにした。ジメチルヒドラジン(DMH)で誘導された大腸がんの前がんのマーカーでACF(Abberant Crypt Foci)を指標に「クルクミン」と「テトラヒドロクルクミン」の抑制効果の検討を行ったところ、「テトラヒドロクルクミン」の方が「クルクミン」よりも強く抑制することが明らかにできた。この研究から、クルクミンが体の中で効果を示すのではなく、強力な抗酸化物質「テトラヒドロクルクミン」に変換されてがん予防効果を示す、ということを明らかにできた。最近、この「テトラヒドロクルクミン」が強力な腎臓がん予防作用も持つのではないか、と期待されている。鉄のキレート化合物であるFe-NTAをマウスに腹腔内注射すると腎臓がんが誘発され、その原因としてフリーラジカルの生成が証明されている。あらかじめ、「クルクミン」と「テトラヒドロクルクミン」を経口でマウスに与えておき、その後にFe-NTAで酸化傷害を誘導した際の防御効果の検討を行ったところ、やはり「テトラヒドロクルクミン」の方に強い防御効果が見出されている。また、肺がん予防効果も期待されるなど「テトラヒドロクルクミン」のもつがん予防効果はの期待は益々高まってきているが、最近、特に注目されているのが、解毒酵素誘導作用である。われわれは、ニンニクをはじめとする香辛料や香辛野菜に高い「解毒酵素」誘導作用があることを見出している。すなわち、「発がん物質」など「毒性物質」が体内に入ると、肝臓でまず第一相の薬物代謝系による活性化を受け、続いての第二相で「抱合反応」とよばれる「高水溶性代謝物」に変換され、最終的には体外へ排泄されることが知られている。詳細は本書の第４章で内田により紹介されているので省略するが、特に、われわれが注目した解毒酵素は「グルタチオンーS-トランスフェラーゼ」で、最近、発現のメカニズムの遺伝子レベルからの解明にも成功している。この解毒酵素の誘導には、ニンニクやワサビをはじめとするアブラナ科の香辛料や野菜に高い効果がみられ、現在、有効成分の単離・精製を進めているが、「クルクミン」、特に「テトラヒドロクルクミン」に強力な解毒酵素誘導作用があることが見出されている。これらの内容を、オリジナル論文としてまとめたので、次に示しておく。

クルクミン、テトラヒドロクルクミンによる酸化ストレス誘導の腎障害予防効果

Abstract

Protective effects of curcumin (U1), one of the major yellow pigments in turmeric and its derivative, tetrahydrocurcumin (THU1), against ferric nitrilotriacetate (Fe-NTA)-induced oxidative renal damage were studied in male ddY mice. Single Fe-NTA treatment (5 mg Fe/kg body weight i.p.) transiently causes oxidative stress, as shown by the accumulation of lipid peroxidation products and 8-hydroxy-2'-deoxyguanosine (8-OHdG) in the kidney. Mice were fed with a diet containing 0.5 % U1 or THU1 for four weeks. U1 and THU1 exhibited significant inhibitory effects on lipid peroxidation and 8-OHdG formation in the kidney. The inhibition by THU1 was stronger than that of U1. To elucidate the mechanisms of protection by U1 and THU1, pharmacokinetics and radical-scavenging capacity of U1 and THU1 were investigated by HPLC and electron spin resonance (ESR) spin trapping with 5,5-dimethyl-1-pyrroline-N-oxide, respectively. Induction of antioxidant enzymes was also investigated. The amounts of THU1 and its conjugates (as sulfates and glucuronides) in the liver and serum were larger in the THU1 group than the U1 group. The amounts of U1 and its conjugates were small even in the U1 group. These results suggest that THU1 be more easily absorbed from the gastrointestinal tract than U1. Furthermore, the THU1 diet maintained or induced antioxidant enzymes such as glutathione peroxidase, glutathione S-transferase and NADPH: quinone reductase better than the U1 diet, and also scavenged Fe-NTA-induced free radicals *in vitro* better than the U1 diet. These results suggest that U1 is converted to THU1 *in vivo* and that THU1 is a more promising chemopreventive agent.

(250 words)

KEY WORDS: • ***curcumin*** • ***tetrahydrocurcumin*** • ***lipid peroxidation*** • ***oxidative stress***

Introduction

Several lines of evidence indicate that oxidative stress may play an important role in various pathological conditions including cancer, neurodegeneration, atherosclerosis, diabetes and rheumatoid arthritis, as well as drug-associated toxicity, postischemic reoxygenation injury, and aging (Halliwell and gutteridge 1999). An iron chelate, ferric nitrilotriacetate (Fe-NTA), induces acute renal proximal tubular necrosis, a consequence of free radical-mediated oxidative tissue damage, that eventually leads to a high incidence of renal cell carcinoma in rodents (Okada and Midorikawa 1982, Li et al. 1987, Ebina et al. 1986). It has been shown that the amount of free radical-associated modified molecules as assessed by lipid peroxidation products, aldehyde-modified proteins and a variety of modified DNA bases such as 8-hydroxy-2'-deoxyguanosine (8-OHdG), reaches the maximum as early as 3 hours after single Fe-NTA treatment, and gradually decreases thereafter (Toyokuni et al. 1994, Uchida et al. 1995).

Curcumin is a major yellow pigment in turmeric (the ground rhizome of *Curcuma longa* Linn), which is widely used as a spice and coloring agent in several foods such as curry, mustard, and potato chips as well as cosmetics and drugs. A wide range of biological and pharmacological activities of curcumin have thus far been investigated (Govindarajan 1980, Huang et al. 1992). Curcumin has been a potent inhibitor of mutagenesis and chemically induced carcinogenesis (Azuine and Bhide 1992, Rao et al. 1995, Nakamura et al. 1998). It possesses many therapeutic properties including anti-inflammatory and anticancer activities (Srimal and Dhawan 1973). Curcumin is currently attracting strong attention by its antioxidant potential as well as its relatively low toxicity to rodents. Curcuminoids also exhibited antioxidant activities in some *in vitro* lipid peroxidation systems (Osawa et al. 1995, Sugiyama et al. 1996) and suppressed TPA-induced hydrogen peroxide production and oxidized DNA formation in the mouse epidermis (Huang et al. 1997). Curcumin has been an inhibitor of neutrophil responses (Srrivastava 1989) and of superoxide (O^{2-}) generation in macrophages (Joe and Lokesh 1994).

Tetrahydrocurcumin (THU1, Fig. 1), one of the major colorless metabolites of curcumin (U1) in the form of glucuronide conjugate, exhibited stronger antioxidant activity than curcumin in several *in vitro* systems (Osawa et al. 1995, Sugiyama et al. 1996). Therefore, THU1 has been hypothesized to be one of the major metabolites with higher physiological and pharmacological activities than U1 in the intestine. However, there is a scarcity of data on the metabolism and antioxidant functions of U1 and THU1 *in vivo*. Furthermore, there is a controversy on which molecule could work as a better chemopreventive agent. THU1 has recently been reported to be a less effective chemopreventive agent in the mouse skin than curcumin (Huang et al. 1997, Conney et al. 1997). However, feeding 0.5 % THU1 in the diet significantly inhibited 1,2-dimethylhydrazine-induced mouse colon carcinogenesis while the inhibitory effect of U1 was not statistically significant (Kim et al. 1998).

In the present study, we fed mice with a diet containing 0.5 % U1 or THU1, evaluated their effects on the Fe-NTA-induced oxidative renal injury, focusing on the ability of U1 and THU1 as antioxidants, and studied the metabolism of these compounds.

Materials and methods

Animals and diet — A total of 54 male ddY mice (Shizuoka Laboratory Animal Center, Shizuoka), weighing 25 to 35 g (6 weeks of age) were used. They were housed jointly each group (n=6) in plastic cages at a temperature of 23 ± 2 °C and an alternating 12h/12h light and dark cycle. All the mice were allowed free access to the diet and deionized water (Millipore Japan, Osaka) for 1 week to adapt themselves to a new environment. The mice were divided into three diet groups of 18 mice each and were fed control or experimental diets containing 0.5 % U1 or 0.5 % THU1 (Table 1) *ad libitum*. Each group of mice were further divided into 3 groups of 6 animals each: untreated control, 1 h or 3 h after Fe-NTA treatment. One month after starting each diet, mice were killed by cervical dislocation at 1 h

or 3 h after Fe-NTA treatment. Blood was taken from the abdominal aorta and the serum was separated. The liver and both kidneys of each animal were immediately removed. The kidneys were homogenized with a Teflon homogenizer in 10 vol. of 50 mmol/L sodium phosphate buffer (pH 7.2). The homogenate was centrifuged at 10,000 x g for 10 min and the supernatant as used for the enzyme activity and thiobarbituric acid (TBA) assay. The supernatant was centrifuged at 105,000 x g for 60 min to obtain the microsome fraction, while the supernatant was taken as the cytosolic fraction.

Materials — U1 and THU1 were a kind gift from Nikken Fine Chemicals Co., Ltd. (Shizuoka), THU1 (> 99 % pure) was prepared from U1 (> 99 % pure) obtained from the rhizomes of turmeric by hydrogenating the two double bonds conjugated to the ß-diketone (Fig. 1). Ferric nitrate enneahydrate and sodium carbonate were from Wako (Osaka) and nitrilotriacetic acid (NTA) disodium salt was from Nacalai Tesque Inc. (Kyoto). The protein concentration was measured using the BCA protein assay reagent obtained from Pierce (Rockford, IL). All the chemicals used were of analytical quality.

Preparation and injection of Fe-NTA — The Fe-NTA solution was prepared immediately before use as previously described (Toyokuni et al. 1994). Briefly, ferric nitrate enneahydrate and the nitrilotriacetic acid (NTA) disodium salt were each dissolved in deionized water to form 300 and 600 mmol/L solutions. They were mixed at the volume ratio of 1:2 (molar ratio, 1:4) and the pH was adjusted with sodium hydrocarbonate to 7.4. Each animal was given an intraperitoneal injection of Fe-NTA at a dose of 5 mg Fe/kg body weight.

Quantitative analysis of curcuminoids — A JASCO MD-910 multiwavelength detector (Tokyo, Japan) was used with the HPLC instrument. A Devolosil ODS-HG-5 column (0.46 cm o.d. x 25 cm, Nomura Chemical, Aichi, Japan) was used for the analysis. The tissue homogenates or the sera with or without glucuronidase/ sulphatase treatment were used for the analysis (Ahmadi et al. 1995). The enzyme treatment was done as follows: 200 μl of 2 vol. tissue homogenates or sera was treated with ß-glucuronidase (500 U) and sulphatase (40 U) in

10 mmol/L PBS (pH 5.0 containing 20 g/L ascorbic acid, 0.1 g/L EDTA). The analysis was carried out with a mobile phase of acetonitrile / H2O (1 g/L trifluoroacetic acid); (50:50 v/v) at a flow rate of 1.0 ml/min. The peak corresponding to U1 was detected at 430 nm after 13 min and THU1 at 280 nm after 10 min.

Measurement of antioxidative activity — In previous study, single intraperitoneal Fe-NTA treatment (5 mg Fe/kg body weight) caused oxidative stress, monitored by the accumulation of lipid peroxidation products and by the formation of 8-OHdG in the time course study (Okada et al. 1999). The renal TBARS or 8-OHdG content has been shown to reach the highest level 3h or 1h after i.p. injection of Fe-NTA, respectively. Hence, we subsequently assessed the formation of the 4-hydroxy-2-nonenal (HNE)-modified proteins, as one of the major oxidatively modified proteins, in the kidney of mice treated with Fe-NTA. Amounts of TBARS, HNE-modified proteins and 8-OHdG levels were measured by the assay as previous study (Okada et al. 1999).

Electron-spin resonance (ESR) spectral measurement — ESR spectra were measured at room temperature with an ESR spectrometer (JES-TE2000, JEOL, Tokyo, Japan) according to the method of Kawabata et al. (1986) after slight modification. 5,5'-Dimethyl-1-pyrroline-1-oxide (DMPO, 10 μl), a radical trapping agent, was added to 200 μl of each tissue homogenate samples (n=4, 10 g protein/L). Then, Fe-NTA (final conc. 10 mmol/L as Fe) was added and mixed well for 10 min. Formation of the DMPO-trapping radical spectra was calculated as the integrated area of the signal.

Enzyme assays — Glutathione peroxidase (GPx) activity was measured by NADPH oxidation in a coupled reaction system containing t-butyl hydroperoxide and oxidized glutathione (Tappel 1978). Assays of NADPH:quinone reductase (NADPH:QR) activity was determined by a procedure reported by Benson et al. (1980) after slight modification. The glutathione S-transferase (GST) activity towards 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate was measured according to the method of Habig et al. (1974), and GST activity toward 4-HNE was measured according to the method of Alin et al. (1985). Catalase (Suzuki

et al. 1993) and superoxide dismutase (SOD) (Beutler 1975, Aebi, 1974) activities were determined as described.

Statistical analysis — Data are expressed as means ± SD. Statistical analysis was performed by means of a two-way analysis of variance (ANOVA). All post hoc multiple comparisons were made with the Scheffe test. The statisitical significant level was set at 5 % ($P < 0.05$). The StatView software (StatView J-4.5; Abacus Concepts, Berkeley, CA) was used for the analysis in each case.

Results

Body weight, and weight of liver and kidney in mice — Body weight and weight of liver and kidney were not different among the three diet groups of mice (data not shown), suggesting that the U1 and THU1 diets were not toxic and did not have bad effect upon the feeding of mice.

Concentration of U1 and THU1 in sera and tissues — In each organ and serum, majority of the U1 and THU1 was found to be present as conjugated glucuronides or sulfates; only a small amount of free form was detected (Table 2, 3). A small amount of U1 and its conjugates (as sulfates and glucuronides) were found to be present in the serum for the U1 diet group, and it was not detected in the liver and kidney. The THU1 concentrations and its conjugates were larger than those of U1 in the liver and serum in the U1 diet group. These results clearly showed that when curcumin was fed, it is transformed into metabolite tetrahydrocurcumin. The concentrations of THU1 and its conjugates in the mice liver and serum were higher in THU1 diet group than U1 diet group (Table 3). The U1 and THU1 were not detected in the kidney because of the limited sensitivity of HPLC analysis.

Effects of U1, THU1 on oxidative stress in the kidney of mice treated with Fe-NTA — As shown in Fig. 2, U1 or THU1 diets significantly suppressed the increase in lipid peroxidation and oxidative modification of DNA induced by Fe-NTA. In addition, the THU1 diet group revealed stronger inhibitory effect on the formation of TBARS and 8-OHdG than the U1 diet group.

TBARS and ESR measurements in the kidney homogenate of mice — Fig. 3 A shows the TBARS contents in the control renal homogenate after treatment with U1 or THU1 at the concentrations indicated, followed by Fe-NTA direct administration. The TBARS contents in the renal control homogenate decreased in a dose-dependent manner. The effect of THU1 was not significantly different from that of U1. Fig. 3 B and C show the ESR spectra of radical spin adducts of DMPO generated from the Fe-NTA treatment in the presence of the U1 and THU1 diet group. Kidney homogenates from the U1 and THU1 diet inhibited the formation of the DMPO-trapping radicals mediated by the Fe-NTA treatment (Fig. 3 C). The THU1 group inhibited the radical generation better than the U1 group.

Enzyme activity in kidney of mice treated with Fe-NTA — Activities of SOD and catalase decreased by Fe-NTA treatment and were also not significantly different between the U1 and THU1 diet groups (data not shown). However, suppression of the GPx activity was less in U1 and THU1 diet groups (Fig. 4 A). Cytochrome P450 activity, the phase I detoxification enzyme, was not changed (data not shown), while suppression of the NADPH: QR and GST activities, the phase II detoxification enzymes, was inhibited in the U1 and THU1 diet groups (Fig. 4 B-D). Especially in the case of the THU1 diet group, the phase II enzymes were more clearly induced than in the U1 diet group. The THU1 diet not only inhibited the decrease in the whole GST activity by Fe-NTA treatment, but also induced stronger GST activity toward HNE than those of the untreated control kidney sample.

Discussion

Recently, there is an increasing interest in the protective function of dietary antioxidants, which are candidates for cancer chemoprevention and for extending the life span. Several antioxidants such as vitamin E, vitamin C, ß-carotene, uric acid, ubiquinols, and flavonoids have been found to play an important role in the nonenzymatic protection against oxidative stress. However, it has been pointed out that one component in these antioxidants is not enough to prevent carcinogenesis. Therefore, a small amount of components in food materials are anticipated to have effects on prevention of carcinogenesis. In our recent studies, when given by oral administration, U1 compounds were observed to be converted to the relational family form of tetrahydro types that had a strong antioxidant activity (Osawa et al. 1995, Sugiyama et al. 1996) in the intestinal tubes. These changes were also detected in cultured cells (Nakayama et al. 1997). We anticipated that THU1 has effect of anti-cancer.

The results of our studies suggest that THU1 has better absorption properties than U1 (Table 2, 3). If the same amount of THU1 diet was given as that of U1, the amounts of THU1 and its conjugates (as sulfates and glucuronides) were larger in the THU1 group than the U1 group. In contrast, the amounts of U1 and its conjugates were small in the both groups. THU1 had the same level of antioxidant capacity as U1 *in vitro* (Fig. 3 A). However, THU1 diet was more effective than U1 diet. Indeed, ESR spectra signal of the radical spin adducts of DMPO mediated by Fe-NTA was more reduced in the THU1-mixed kidney homogenate than in the U1-mixed homogenate (Fig. 3 B, C).

Preferential induction of phase II biotransformation enzymes such as glutathione S-transferases (GSTs) and NAD(P)H:quinone reductase (as opposed to phase I biotransformation enzymes of the cytochrome P-450 systems) has been suggested to be one possible mechanism for the effects of a number of antioxidants towards cancer prevention (Song et al. 1999, Wattenberg 1985, Talalay 1989). Orally administered U1 has been shown to slightly

increase GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) in mouse liver even with a high dose (Susan and Rao 1992). However, GST activity towards HNE has a relatively high activity in the mouse liver (Piper et al. 1998). GSTs consist of several catalytically distinct isozymes, each of whose expression is differentially regulated by the oxidant or antioxidant environment within the cell (Hayes and pulford 1995). Therefore, the present studies were designed to investigate the effects of oral U1 and THU1 administration on GSH-linked antioxidant defenses including GPx activity, GST activities towards CDNB and towards HNE in the mouse kidney after Fe-NTA injection.

We found that the antioxidant effects of U1 was augmented through restoration of decreasing GPx and the HNE metabolizing GST isozymes activities by Fe-NTA (Fig. 4). Also, the effect of THU1 was stronger than U1 in these antioxidant enzyme inductions. In the present study, it was also shown that there was a significant effect of oral THU1 exposure during phase II enzyme induction (Fig. 4). Namely, in the THU1 group, the GST activity towards HNE was induced more than the control group. This induction might result from THU1 exposure alone. More detailed study to clarify the molecular mechanism how these antioxidants work for enzyme induction is currently in progress.

Phase I enzymes inactivate an alien substance by a redox reaction and hydrolysis. Cytochrome P450, one of the major phase I enzymes, is thus induced by foreign factors such as drugs, industrial chemical substances, food additives, tobacco, alcohol, various food factors. However, a variety of chemical substances are also activated by cytochrome P450. Compounds of this kind have been classified either as bifunctional inducers, which elevate both the phase I and phase II enzymes, or as monofunctional inducers, which selectively elevate the phase II enzymes. The induction of the phase I enzymes, such as cytochrome P450 isozymes, is required for the metabolic disposal of xenobiotics (Williams 1967), but is also considered as a risk factor due to the potential of activating procarcinogens (Yang 1994). Therefore, the finding of U1 and THU1 as monofunctional inducers in foods gave them a biologically important merit.

In conclusion, the present study provided clear evidence for the suppression of oxidative stress-induced renal damage by dietary administrated U1 and THU1. U1 and THU1 are probably working in two different ways: 1) direct chelating or scavenging effects and 2) induction of the antioxidant enzymes (monofunctional inducers). The effect of THU1 was greater than that of U1 as an antioxidant *in vivo*. It might be suggested that THU1 is more easily absorbed than U1 from the gastrointestinal tract. It may be important to note that THU1 has some advantages for the application to food additives because it is colorless and yet is easy to be prepared by the standard hydrogenation of U1. Further studies of curcuminoids on oxidative stress, especially on their molecular mechanisms, are necessary. We believe that THU1 has a robust potential of contributing to the chemopreventive application in humans.

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Legends to Figures

Fig. 1. Chemical structures of curcumin and tetrahydrocurcumin

Fig. 2. Effect of curcumin and tetrahydrocurcumin on ferric nitrilotriacetate (Fe-NTA)-induced oxidative stress suppression in the kidney of mice. Mice were intraperitoneally treated with Fe-NTA (5 mg Fe per kg body), and oxidative stress was monitored by the

formation of 2-thiobarbituric acid reactive substances (TBARS) (A), 4-hydroxy-2-nonenal (HNE)-modified proteins (B) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) (C). Data are expressed as means ± SD, n=6. Different superscript letters are significantly different as determined by two-way ANOVA and the Scheffe post hoc test ($P < 0.05$).

Fig. 3. Effect of curcumin and tetrahydrocurcumin on lipid peroxidation and Electron-spin resonance (ESR) spectra of 5,5'-Dimethyl-1-pyrroline-1-oxide (DMPO) spin adducts in the *in vitro* experiments with control kidney homogenates after ferric nitrilotriacetate (Fe-NTA) addition. A, Lipid peroxidation in mouse kidney samples which was incubated with various concentrations of U1 and THU1 (0 to 100 μmol/L), ascorbic acid (100 μmol/L) and Fe-NTA (10 μmol/L). B, ESR spectra of DMPO spin adducts formed. Twenty microliters of DMPO and Fe-NTA were added to 200 μl of mouse tissue homogenate. ESR spectra were recorded 1 min after DMPO addition. C, Relative spin area of ESR spectra compared with control homogenates without Fe-NTA. Data were presented as percent control. Data are expressed as means ± SD, n=4. Different superscript letters are significantly different as determined by two-way ANOVA and the Scheffe post hoc test ($P < 0.05$).

Fig. 4. Effect of curcumin and tetrahydrocurcumin on glutathione peroxidase (GPx), NADPH:quinone reductase (QR) and glutathione S-transferase (GST) activities in the kidney of mice treated with ferric nitrilotriacetate (Fe-NTA). A, GPx activity. B, QR activity. C, GST activity towards 1-chloro-2, 4-dinitrobenzene (CDNB). D, GST activity towards 4-hydroxy-2-nonenal (HNE). Data are expressed as means ± SD, n=6. Different superscript letters are significantly different as determined by two-way ANOVA and the Scheffe post hoc test ($P < 0.05$).

Table 1

Composition of control and experimental diets

Ingredient	control diet	0.5 % curcumin (U1) diet	0.5 % tetrahydrocurcumin (THU1) diet
		(g/100 g)	
Casein	25.0	25.0	25.0
Cellulose	4.0	4.0	4.0
Corn oil	5.0	5.0	5.0
Mineral mixture[1]	3.5	3.5	3.5
Vitamin mixture[2]	1.0	1.0	1.0
Corn starch	61.5	61.0	61.0
U1	---	0.5	---
THU1	---	---	0.5

[1]Composition of mineral mixture (g/100 g mix): $CaHPO_4 \cdot 2H_2O$, 0.43; KH_2PO_4, 34.31; NaCl, 25.06; Fe-Citrate, 0.623; $MgSO_4$, 4.8764; $ZnCl_2$, 0.02; $MnSO_4 \cdot 5H_2O$, 0.121; KI, 0.0005; $CaCO_3$, 29.29; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.0025; cellulose-powder, 5.1036.

[2]Composition of vitamine mixture (g/100 g mix): retinyl acetate, 0.1; cholecalciferol, 0.00025; all-rac-α-tocopheryl acetate, 0.5; menadion, 0.52; thiamin-HCl, 0.12; riboflavin, 0.4; pyridoxine-HCl, 0.08; vitamin B_{12}, 0.00005; vitamine C, 3.0; biotin, 0.002; folic acid, 0.2; calcium-pantothnate, 0.5; *p*-aminobenzoic acid, 0.5; nicotinic acid, 0.6; choline-chloride, 20.0; cellulose powder, 73.0577.

Table 2

Distribution of curcuminoide in mice fed with curcumin (U1)

Curcuminoid	Liver		Kidney		Serum
	free	conjugate	free	conjugate	free+conjugate
	(nmol/mg)		(nmol/mg)		(μmol/L)
U1	N.D.	N.D.	N.D.	N.D.	0.6 ± 0.1
THU1	N.D.	3.5 ± 0.4	N.D.	N.D.	14.4 ± 3.9

[n =6 , mean ± SD]
N.D. : not detected

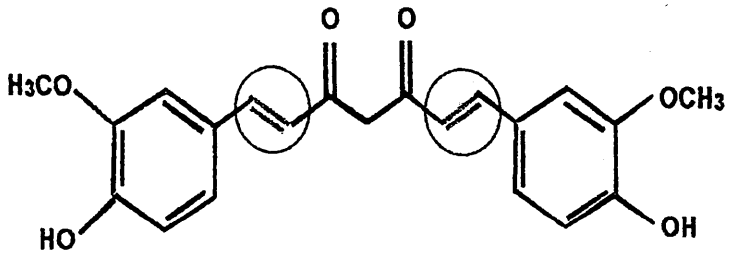
Table 3

Distribution of curcuminoide in mice fed with tetrahydrocurcumin (THU1)

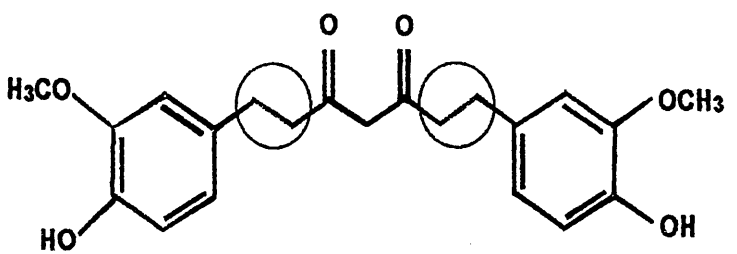
Curcuminoid	Liver		Kidney		Serum
	free	conjugate	free	conjugate	free+conjugate
	(nmol/mg)		(nmol/mg)		(μmol/L)
U1	N.D.	N.D.	N.D.	N.D.	N.D.
THU1	2.5 ± 0.6	7.9 ± 1.6	N.D.	N.D.	43.4 ± 15.5

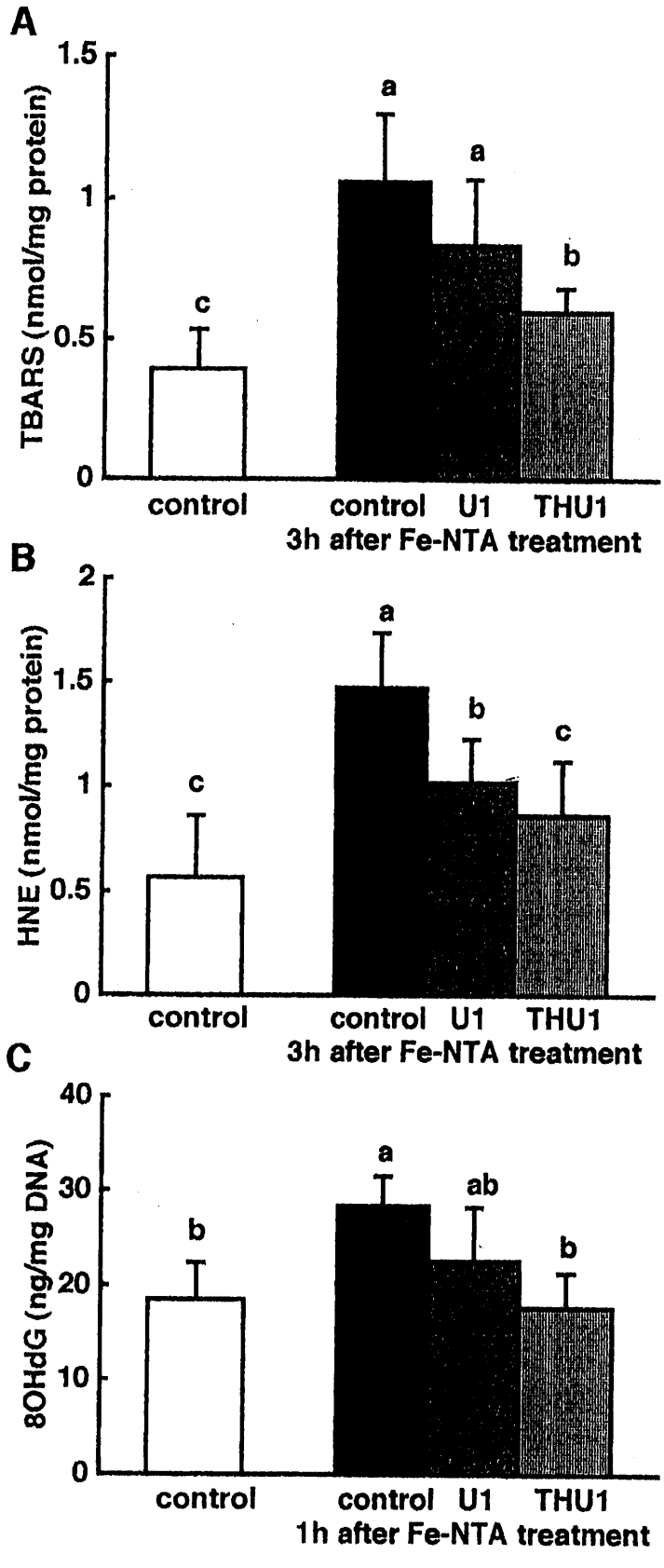
[n =6 , mean ± SD]
N.D. : not detected

Curcumin (U1)

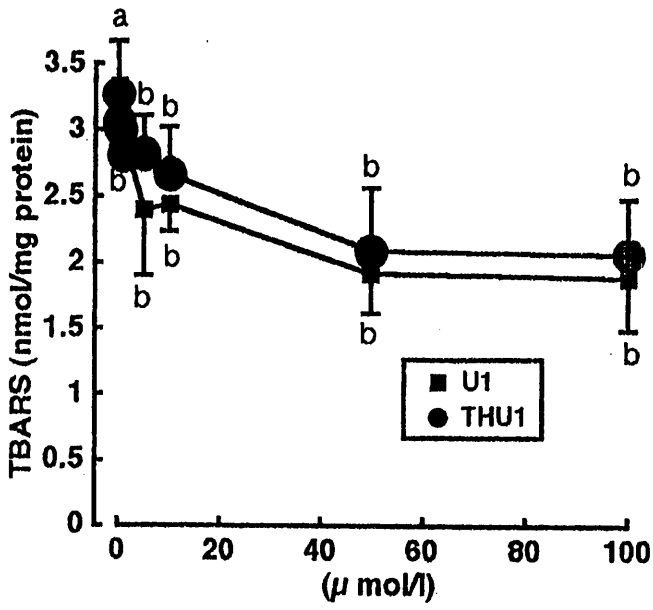


Tetrahydrocurcumin (THU1)

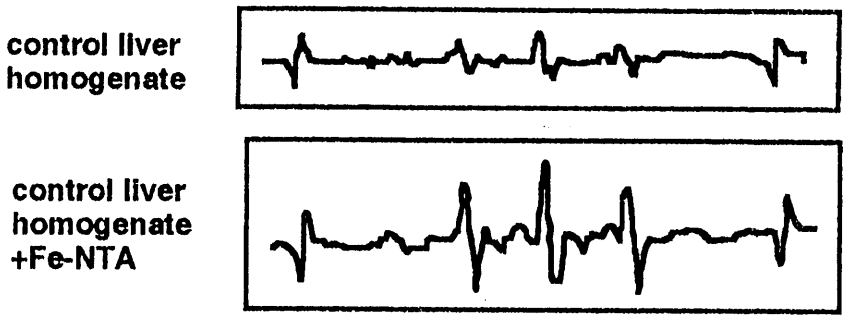



A
TBARS (nmol/mg protein)
1.5
1
0.5
0
a
a
b
c
control
control
U1
THU1
3h after Fe-NTA treatment
B
HNE (nmol/mg protein)
2
1.5
1
0.5
0
a
b
c
c
control
control
U1
THU1
3h after Fe-NTA treatment
C
8OHdG (ng/mg DNA)
40
30
20
10
0
a
ab
b
b
control
control
U1
THU1
1h after Fe-NTA treatment

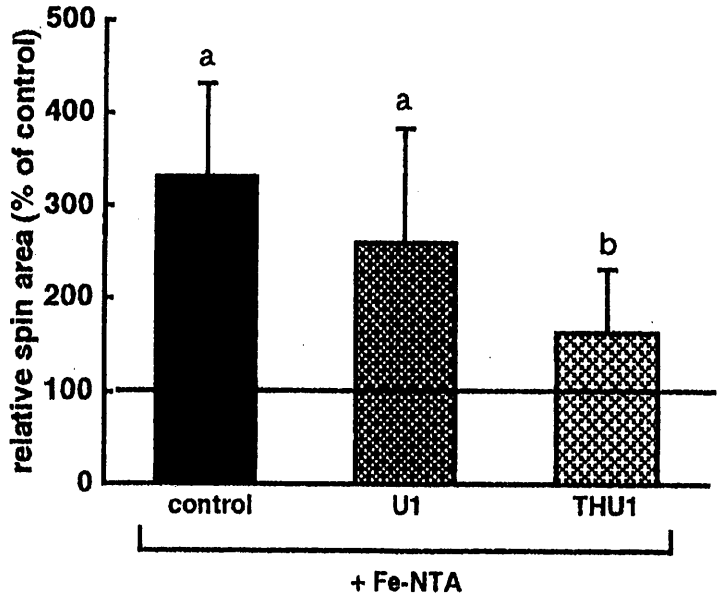
A


TBARS (nmol/mg protein)
3.5
3
2.5
2
1.5
1
0.5
0
0
20
40
60
80
100
(μ mol/l)
a
b
U1
THU1

B


control liver homogenate
control liver homogenate +Fe-NTA

C


relative spin area (% of control)
500
400
300
200
100
0
a
a
b
control
U1
THU1
+ Fe-NTA

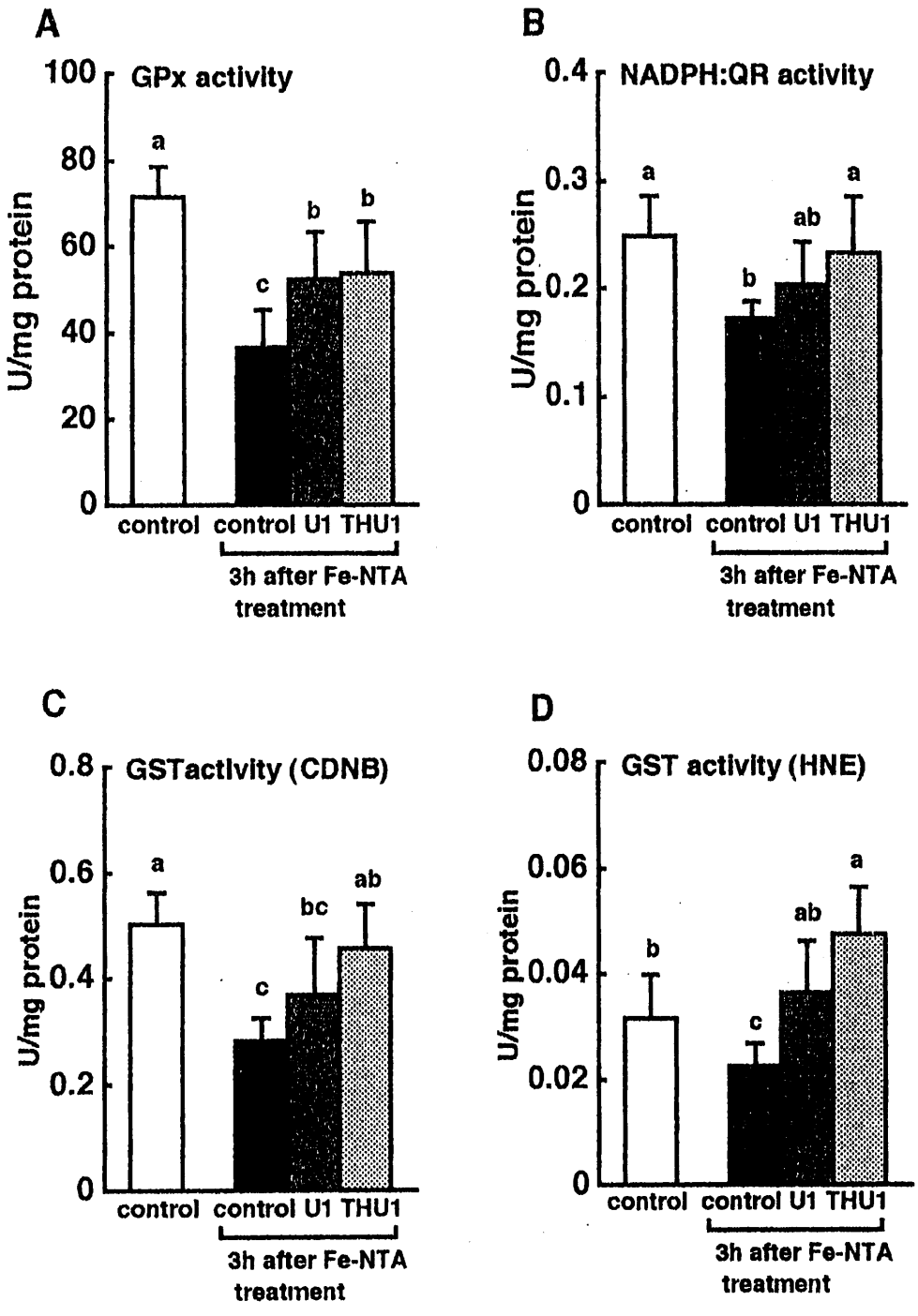

A
GPx activity
U/mg protein
100
80
60
40
20
0
a
b
b
c
control
control U1 THU1
3h after Fe-NTA
treatment
B
NADPH:QR activity
U/mg protein
0.4
0.3
0.2
0.1
0
a
ab
a
b
control
control U1 THU1
3h after Fe-NTA
treatment
C
GSTactivity (CDNB)
U/mg protein
0.8
0.6
0.4
0.2
0
a
ab
bc
c
control
control U1 THU1
3h after Fe-NTA
treatment
D
GST activity (HNE)
U/mg protein
0.08
0.06
0.04
0.02
0
a
ab
b
c
control
control U1 THU1
3h after Fe-NTA
treatment

Figure 4

クルクミン、テトラヒドロクルクミンの糖尿病合併症予防効果については、最近、協和発酵（株）研究グループとの研究を進めたばかりである。予備的な研究であるが、最近、興味ある結果を得ることができたので、概略を紹介する。

われわれが行ったのは、オスのSDラット（n=4）に２５％ガラクトースを含んだ飼料を与え、６週間後に生じた白内障に対する抑制効果の検討を行った。クルクミン、テトラヒドロクルクミンは０．５％餌にまぜて摂取させたところ、６週間後に生じた末期的な白内障は顕著に抑制された（図１）。

そこで、テトラヒドロクルクミンに焦点をあてて、サルを用いたin vitro糖尿病白内障予防効果の検討を行った。水晶体は、３０mMキシロース含有培地で培養し、テトラヒドロクルクミンは最終濃度１００μMとなるように添加した。測定は、水晶体全面積を１００として混濁部面積の割合を混濁度（％）として表示した（図２）。

ガン予防効果や腎不全改善効果は、クルクミンの生体内代謝物であるテトラヒドロクルクミンの方が強く、今回の実験でも、テトラヒドロクルクミンは、ラット、サルの両方の系で、代表的な糖尿病合併症の一つである白内障を効果的に抑制したことは、興味ある結果である。今後、再度、実験を繰り返すとともに、臨床研究も行う予定である。

最後に、本研究の遂行にあたり、研究費の援助を賜った日本学術振興会に深謝する。

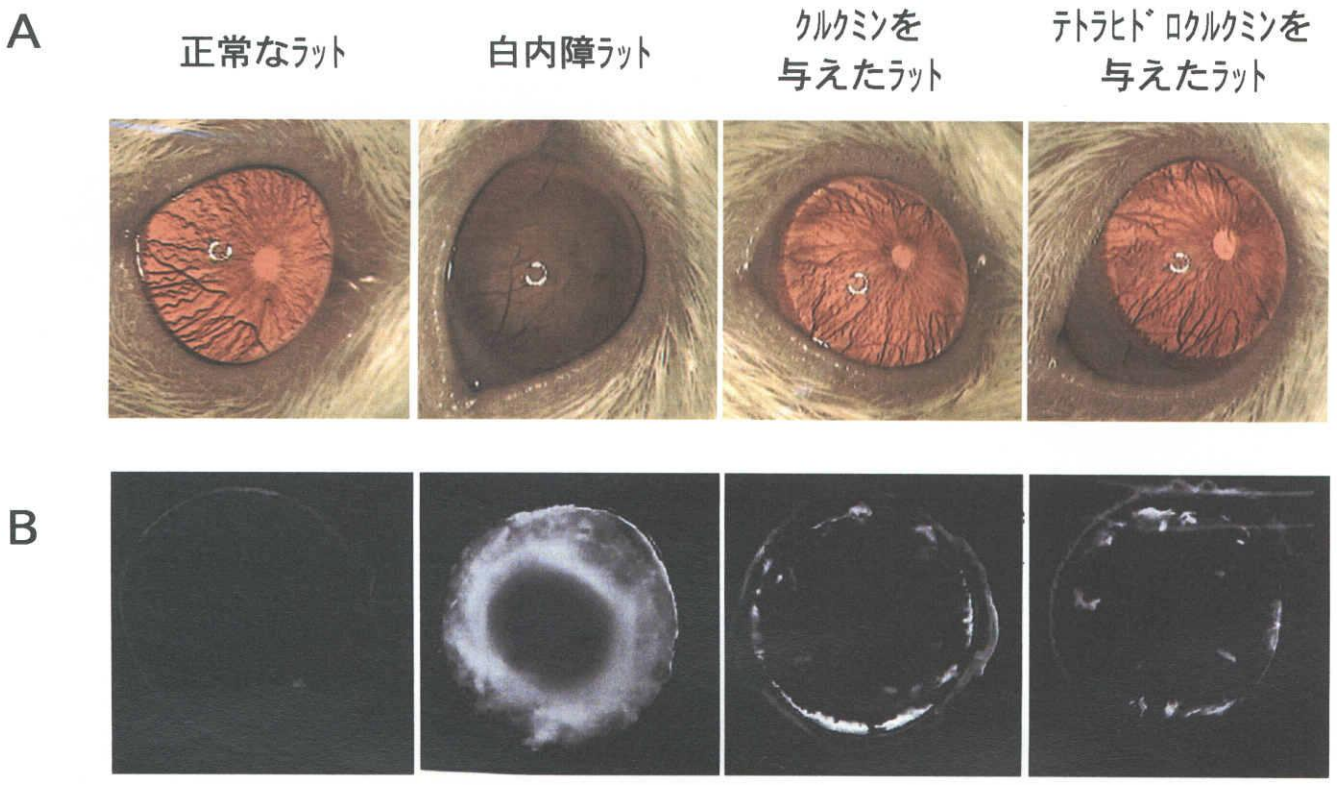
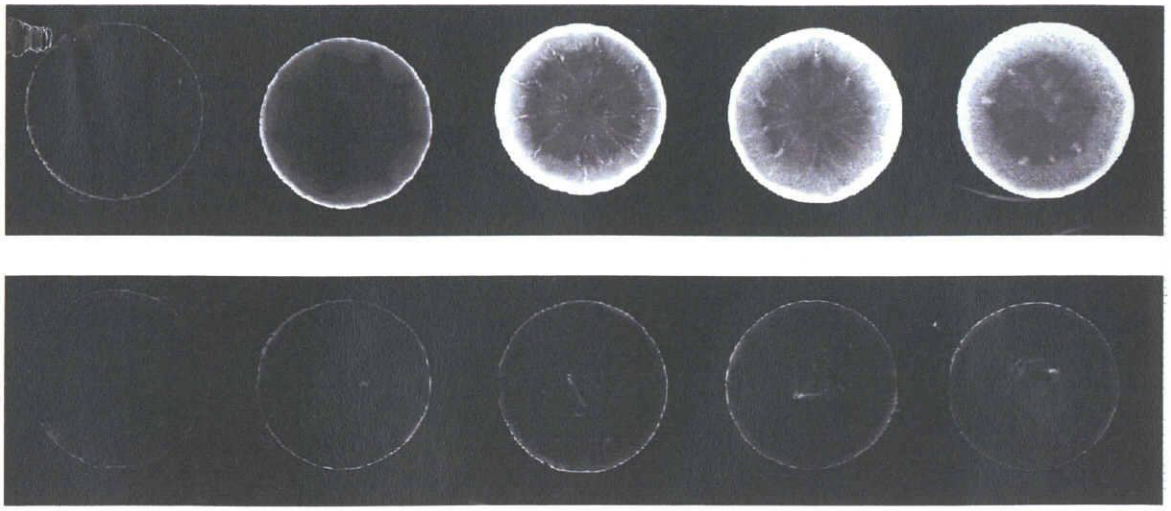


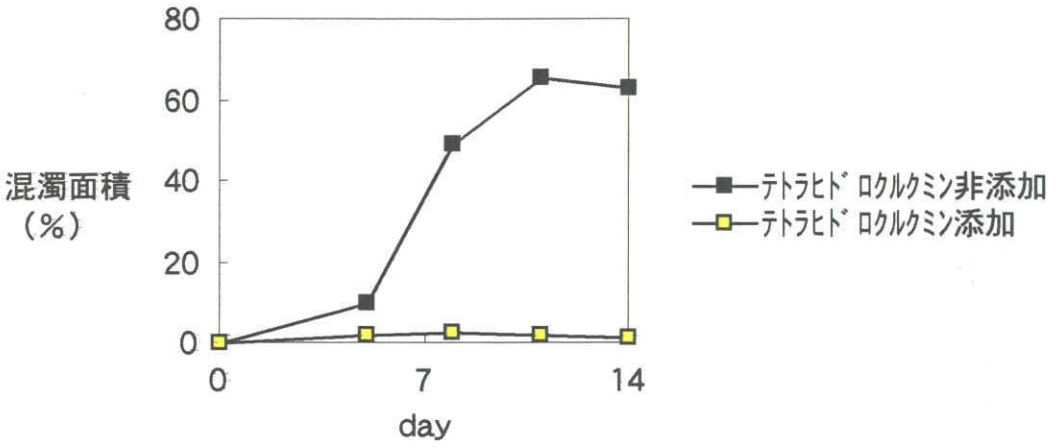
図1　ﾗｯﾄin vivo糖尿病白内障ﾓﾃﾞﾙでのｸﾙｸﾐﾝ類の効果
A: ﾗｯﾄの眼球の外見的所見
B: ﾗｯﾄ摘出水晶体

SDﾗｯﾄ（ｵｽ）に25%ｶﾞﾗｸﾄｰｽを含んだ飼料を与えると6週間後には末期的な白内障が生じたが、ﾃﾄﾗﾋﾄﾞﾛｸﾙｸﾐﾝやｸﾙｸﾐﾝをそれぞれ0.5%餌に混ぜて摂取させると白内障の進行が顕著に抑制された。ここでは典型的な例を示した。（n=4）

A



B



C

	培養開始後（日目）				
	0	5	8	11	14
ﾃﾄﾗﾋﾄﾞﾛｸﾙｸﾐﾝ非添加	0.0	9.7	49.5	65.5	62.7
ﾃﾄﾗﾋﾄﾞﾛｸﾙｸﾐﾝ添加	0.0	1.7	2.7	2.2	1.4

単位：%

図2　ｻﾙ in vitro 糖尿病白内障ﾓﾃﾞﾙでのﾃﾄﾗﾋﾄﾞﾛｸﾙｸﾐﾝの効果

A:（上）30mMｷｼﾛｰｽ含有培地で培養した水晶体
（下）30mMｷｼﾛｰｽ含有培地にﾃﾄﾗﾋﾄﾞﾛｸﾙｸﾐﾝを終濃度100μMで添加して培養した水晶体

B, C: 上記のA, Bについて写真上の水晶体全面積を100とし、混濁部面積の割合を水晶体の混濁度(%)として示した

写真は水晶体の培養後0, 5, 8, 11, 14日後に撮影した。
ｷｼﾛｰｽ含有培地で5日目あたりから水晶体の混濁が観察されたが、
ﾃﾄﾗﾋﾄﾞﾛｸﾙｸﾐﾝを培地中に加えた場合には混濁の進行が抑制された。