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F. 研究発表

1. 論文発表

なし

2. 学会発表

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G. 知的財産権の出願・登録状況（予定を含む）

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

平成17年度 厚生労働科学研究費補助金(化学物質リスク研究事業)
分担研究報告書

研究課題名:ナノマテリアルの安全性確認における健康影響評価手法の確立に関する研究

分担研究課題名:ナノマテリアルの化学物質評価の面からの基礎的調査及び評価法に関する総合的研究

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研究要旨

ヒト健康に及ぼす影響評価する上で未知である点が多いナノマテリアルの安全性評価を確立するうえにおいて、必要な事項についてOECD等の国際的動向を踏まえて整理することを目的とし、17年度は、10月のミネアポリスで開かれた「第2回ナノテクノロジーと職業衛生に関する国際シンポジウム」に参加し、本研究の実験計画や予備的知見について発表すると共に、12月に開かれたOECDの「産業用ナノマテリアルの安全性に関するワークショップ」に出席し、情報を収集した。その結果、まず、暴露状況の把握、物質の同定法の確立、試験サンプルのための標準化等を整備することの重要性とで、*in vivo*試験によってバリデートされた、スクリーニング試験の開発を行う必要がある。そのためには、ADME情報や蓄積(沈着)性やエンドポイントを見極めるための分子レベルでの相互作用等の解析に関した情報が重要な事項となると考えられた。

A. 研究目的

ナノテクノロジーは、「ナノメートルサイズのスケールで原子や分子を自由に操作・制御し、物質の構造・配列を制御することで、新機能や優れた特性を持つ物質を作り出す技術」とされ、国家戦略としてその開発が進められている。この中でも、フラーレンやカーボンナノチューブに代表されるナノサイズの新素材であるナノマテリアルは薬物輸送を含む医療への展開を初めとした各種の応用が急速に進んでいる。他方、ナノサイズの物質は、タバコの煙、ディーゼル排気、などに非意図的に発生することが従来知られているが、その成分が混合物であり分子構造が不明であったりすることから、その中のナノ粒子サイズ物質の有害性については、直接的に取り上げられることが少なく、ナノマテリアルの生体影響については、多くの点で未知である。近年、神経系や免疫系への影響やDNA障害などの懸念を示す報告はされているものの、その物性を適切に考慮した評価研究はほとんど行われていない状況である。また、評価上の問題点として、様々な種類のナノマテリアルやその用途があり、ナノマテリアルの生物学的影響がこれらの多様性に影響されると考えられることから、一様に取り扱うことがでないこと、さらにナノマテリアルの環境中および生体中の測定法も確立していないことが挙げられる。したがって、どの程度の暴露リスクがあるのかについて、用途情報も含めて不足している他、生体内への吸収や分布についての情報も少ない。

そこで、本研究では、ナノマテリアルのヒトに与える健康影響のリスクを評価する上で、必要な事項についてOECD等の国際的動向を踏まえて整理することを目的とする。

B. 研究方法

17年度は、10月のミネアポリスで開かれた「第2回ナノテクノロジーと職業衛生に関する国際シンポジウ

ム」に参加し、本研究の実験計画や予備的知見について発表した。また、12月に開かれたOECDの「産業用ナノマテリアルの安全性に関するワークショップ」に出席し、情報を収集した。

C. 研究結果

現在までに報告されている断片的な毒性情報の状況から、ナノマテリアルの安全性確認に必要な生体影響試験に関して必要な事項としては、まずナノマテリアルの体内動態を様々な暴露経路や状況に応じて把握することが必要であると考えられる。この観点に立って、暴露評価とADME(吸収・分布・代謝・排泄)の観点から留意すべき事項について整理した。

暴露評価

- 粒子サイズの同定、暴露濃度／量(暴露環境は粒子サイズに依存する物理・化学的性状や濃度、分散状態に大きく依存する)
- ナノマテリアルが実際の製品中(ポリマーなど)に含有されている場合は同時に暴露される可能性を考慮する必要がある
- 環境暴露を考慮した場合、製品等の使用中あるいは廃棄後、環境中での修飾・分解産物の考慮も必要

ADME評価

- 吸収:粒子サイズや暴露媒体の種類に依存して吸収率が影響を受ける可能性
- 分布:粒子サイズに依存した物理化学的性状や細胞による貪食作用を考慮する必要がある。また、組織－血管関門、脳－血液関門や胎盤輸送などの可能性を考慮する。特定の組織や細胞への沈着の可能性
- 代謝:毒性活性化体への体内での変換。生体内高分子や外来化学物質との相互作用の可能性
- 排泄:排泄組織(腎臓や膀胱など)での沈着の可能性(物性に依存した集合体の形成などにより)。

毒性評価に必要な事項

- 標準化された投与(暴露)手法が必要。
- 投与(暴露)時の粒子サイズの制御)(*in vivo*)、培地への溶解性 (*in vitro*)
- これらは、実際の暴露状況を反映したものであることが望ましい(暴露評価の情報が必要)
- 数多くの物質を評価するためには、*in Vitro*によるスクリーニング系の開発も必要だが、その前にどのような有害性がどの暴露経路で現れるかを検索しておく必要がある。(個別の有害影響毎のスクリーニング系が必要)
- 生体内組織での長期間にわたる沈着が疑われる場合は、慢性試験も必要
- 分子レベルでの生体分子との反応性やそのメカニズム解析(新しい試験系の開発と共に、遺伝子導入／ノックアウト動物を用いた解析やオミクス技術(トキシコゲノミクスなど)を用いた先端的解析手法の導入が有用)

OECDのワークショップ報告:

国内外共に、1990 年代から特に医療技術開発の中で個々の成果物に対して個別的に生体影響等が検討されてきているところであるが、ナノマテリアル全体としての社会影響や安全性に関する検討の動きは 2000 年ごろに始まる。2000 年の米国国家ナノテクノロジー戦略(NNI)では、当初から社会影響が念頭に置かれており、一方 EU でも 2001 年からの NANO-PATHOLOGY Project や 2003 年からの NANODERM および NANOSAFE の各プロジェクトによってリスク評価に向けた動きが開始されてきていた。その中で 2004 年頃から、ナノマテリアルの中心的存在であるカーボンナノチューブやフラーレンに対する *in vivo* 試験において有害性を示唆する報告がなされ、ナノマテリアル全体の健康影響問題が注目を浴びるようになり、2005 年にかけてこの問題を扱った国際シンポジウムやワークショップが数多く開催されている状況である。2005 年に開かれた日本学術会議と英国王立協会共催の日英ワークショップでは、国際的な情報交換や共同研究の必要性と共に毒性試験の標準化の必要性が提唱されているところでもある。ILSI のワーキンググループでは *in vitro* 試験法と共に実際の

暴露環境に応じた *in vivo* 試験法の開発が望まれるとされた。こうした状況の中 12 月には OECD の「産業用ナノマテリアルの安全性に関するワークショップ」が開催され、物性・標準化、環境影響、健康影響、規制関係に分かれた討論が行われ、健康影響評価手法について以下に示すような討論が行われた。

- すべてのナノマテリアルについてフル毒性試験は不可能であるだろう。しかし、すべてのナノマテリアルについて、いくつかの毒性の可能性を示唆するストラテジーが必要である。これらのストラテジーはデータが蓄積にした時にはリファインされるべきである。
- 選別された一連のナノマテリアルについての深い洞察が必要。個々のナノマテリアルに対する毒性試験(あるいは優占付け)をナノマテリアル全体に対する理解、(将来カテゴリ化あるいは一般化するための) に繋げるために
- ILSI の報告で述べられたような段階的アプローチあるいは決定樹のようなものが推薦される。あるナノマテリアルにもっとも適切な毒性試験手法を決定するために、短期 *in vivo* 試験、補助的な *in vitro* 試験は慢性毒性の可能性を示唆すべきである。生物学的消失(ADME、persistence 蓄積性、)は重要な事項であるかもしれないし、これらの考察に対する gap でもあるかもしれない
- 試験のストラテジーのゴールは、*in vitro* 試験やコンピュータシミレーションのような、スクリーニング試験を開発することであるべき。それらスクリーニング試験は *in vivo* 試験法によってバリデートされたものであるべきである。
- いくつかの OECD ガイドラインは修正されるべきかもしれない。そしてナノマテリアルの評価を補助するための新しい試験法も必要かもしれない。
- 試験されたマテリアルの最小の標準化された物理学的キャラクタリゼーションは必要
- ナノマテリアルの国際的に調和された標準レファレンスの供給体制の設立が必要
- ナノマテリアルの毒性試験を行うときには、ナノマテリアルのダイナミックな性質を考慮する必要がある。(表面コート、aggregation 集合化／disaggregation 、 agglomeration 凝集化/deagglomeration を含む性質について)
- ナノマテリアルのバリアントにレンジの存在する可能性は、さらなる毒性試験にとってのチャレンジである。

全体的なワークショップの提言としては、物性・標準化、環境影響、健康影響、規制関係の様々なグループでの討論の結果、今後 OECD においてワーキンググループの設置を求めるという方向になった。

D. 考察

有害性評価において、有害性を検討する前に、暴露状況の把握、物質の同定法の確立、試験サンプルのための標準化等を整備することは重要である。また、理想的には慢性影響も含めたフル毒性試験が必要ではあるが、現実的には *in vivo* 試験によってバリデートされた、スクリーニング的 *in vitro* 試験(やコンピュータシミレーション)の開発を行う必要がある。そのためには、ADME 情報や蓄積(沈着)性やエンドポイントを見極めるための分子レベルでの相互作用等の解析に関した情報が重要な事項となると考えられる。そしてこれらを使った効率的な有害性評価のためには、段階的アプローチや決定樹を用いたような評価システムの構築が有用であると考えられる。

本研究では生産量の高い物質と言う理由で、酸化チタンやフラーレンを中心に展開してきたが、多層型カーボンナノチューブについても品質的に均一なもので急速な産業展開が行われようとしているという情報を入手した。これは、日本学術会議－英国王立協会共同プロジェクトの「ナノテクノロジーの健康、環境、社会的影響に関するワークショップ」に関する活動過程で入手したものであるが、すでに一部は評価サンプルとして、研究機関への提供が行われている。粒子形状がアスベストに近いと考えられている多層型カーボンナノチューブ

(MWCNT)の産業的な展開の加速は、in vivo 系での慢性生体影響評価手法の開発を中心とした研究展開の緊急強化の必要性を示していると考えられる。

E. 結論

ナノマテリアルのヒト健康に及ぼす影響評価する上で、必要な事項について OECD 等の国際的動向を踏まえて整理した結果、まず、暴露状況の把握、物質の同定法の確立、試験サンプルのための標準化等を整備することの重要性とで、*in vivo* 試験によってバリデートされた、スクリーニング試験の開発を行う必要がある。そのためには、ADME 情報や蓄積(沈着)性やエンドポイントを見極めるための分子レベルでの相互作用等の解析に関した情報が重要な事項となると考えられた。

F. 研究発表

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G. 知的財産権の出願・登録状況(予定を含む)

1. 特許取得
(該当なし)
2. 実用新案登録
(該当なし)
3. その他
(該当なし)

別添５

Ⅲ．研究成果の刊行に関する一覧表

書籍

著者名	論文タイトル	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ

雑誌

著者名	論文タイトル	発表誌名	巻号	ページ	出版年
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SEMI-QUANTITATIVE IMMUNOHISTOCHEMICAL ANALYSIS OF MALE RAT-SPECIFIC α_{2u}-GLOBULIN ACCUMULATION FOR CHEMICAL TOXICITY EVALUATION

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ABSTRACT — We purified male rat urinary α_{2u}-globulin, prepared the antibody in rabbits, and improved an immunohistochemical detection method using this antibody for male rat-specific α_{2u}-globulin accumulation appearing as hyaline droplets in the kidneys. Our prepared antibody reacted specifically with α_{2u}-globulin in both immunohistochemical and Western blotting analyses, furthermore, and the graded immuno-reactivities on the slide were well associated with computational image analyzing results. Using this method, we retrospectively analyzed the renal sections from the toxicity studies of 12 nephrotoxic chemicals, which had already been conducted under the Japanese Existing Chemicals Survey Program. We demonstrated that the hyaline droplets induced by treatment with 10 chemicals (1,4-dibromobenzene, dicyclopentadiene, 3,4-dimethylaniline, 1,4-dicyanobenzene, tetrahydrothiophene-1,1-dioxide, 1,3-dicyanobenzene, acenaphthene, 3,4-dichloro-1-butene, 3a,4,7,7a-tetrahydro-1H-indene and 3,5,5-trimethylhexan-1-ol) were directly associated with α_{2u}-globulin accumulation. This immunohistochemical method is convenient for applying, even retrospectively, paraffin sections from general toxicity studies and could be useful for qualifying male rat-specific hyaline droplets consisting of α_{2u}-globulin and renal risk in humans.

KEY WORDS: α_{2u}-globulin, Immunohistochemistry, Hyaline droplet, Nephrotoxicity

INTRODUCTION

For risk assessment of chemicals, the most critical data are derived from animal toxicity studies because of a general lack of information on humans. Although all available results from animal studies have been applied to human risk assessment, in principle, exclusion of some specific toxicities, which might not occur in humans, should be taken into account. Among laboratory animals, the rat has been commonly used for toxicity studies, especially sub-acute, long-term or carcinogenicity studies. Nephropathy with hyaline droplets and renal tubular neoplasia caused by chemicals inducing α_{2u}-globulin accumulation (CIGA) are considered to be a male rat-specific toxicity, not occurring in female rats or other animals, including primates. Although low molecular proteins homologous to α_{2u}-globulin can be detected in other species, including mice and humans, none of these proteins have been confirmed to bind to CIGA, followed by accumulation of the protein-CIGA complex as in the case of α_{2u}-globulin. It is therefore believed that renal toxicity induced by CIGA in male rats is unlikely to occur in humans (Hard *et al.*, 1993).

α_{2u}-Globulin was first identified in male rat urine (Roy and Neuhaus, 1966), and had been reported to be a male rat-specific protein with a molecular weight of 18 to 20 kDa. The major source of urinary α_{2u}-globulin

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is the liver, where α_{2u}-globulin mRNA constitutes approximately 1% of the total hepatic mRNA (Sippel *et al.*, 1976; Kurtz and Feigelson, 1977). Neither α_{2u}-globulin nor its mRNA is detectable in the female liver (Sippel *et al.*, 1975, 1976; MacInnes *et al.*, 1986). The blood α_{2u}-globulin secreted from the liver is freely filtered through the glomerulus, and in mature rats, about two-thirds of the filtered protein is reabsorbed by tubules and the remainder is excreted through the urine (Neuhaus *et al.*, 1981). CIGA binds noncovalently to α_{2u}-globulin, and the resulting complex shows less degradability with proteolytic enzymes, causing an accumulation of the complex that is detectable as hyaline droplets with a light microscope. Various chemicals have been suspected of being CIGA based on detection of the evidence for exacerbation of hyaline droplets in renal proximal tubules in male rats, though not in females. Direct evidence for increasing α_{2u}-globulin levels has been demonstrated for only a few of these chemicals, however, including 2,2,4-trimethylpentane (Stonard *et al.*, 1986; Charbonneau *et al.*, 1987; Lock *et al.*, 1987), decalin (Kanerva *et al.*, 1987), d-limonene (Lehman-McKeeman *et al.*, 1989; Webb *et al.*, 1989), 1,4-dichlorobenzene (Charbonneau *et al.*, 1989), isophorone (Strasser *et al.*, 1988), lindane (Dietrich and Swenberg, 1990), tri- or per-chloroethylene and pentachoroethane (Goldsworthy *et al.*, 1888).

A number of initial safety assessments has so far been conducted for industrial chemicals, including both new and existing chemicals by the Japanese government or the OECD high production volume chemicals programs. Certain chemicals among these industrial chemicals have been suspected of being CIGA. In some cases, however, renal changes in male rats have been assessed as the endpoint for extrapolation to human health risk owing to a lack of direct evidence caused by α_{2u}-globulin accumulation, because no antibody against α_{2u}-globulin is commercially available for general toxicity studies. Some immunohistochemical α_{2u}-globulin analysis methods had already been developed (Burnett *et al.*, 1989; Hashimoto and Takaya, 1992; Caldwell *et al.*, 1999). As these methods required glycolmethacrylate embedding or specific computational analysis, they would be inappropriate for confirming α_{2u}-globulin accumulation in routinely conducted guideline-based toxicity studies. We therefore improved an immunohistochemical α_{2u}-globulin detection system using paraffin sections, which are generally used for standard toxicity studies. We evaluated the several chemicals suspected of being CIGA, moreover, and indicated the direct evidence caused by α_{2u}-globulin accumulation.

MATERIALS AND METHODS

Preparation of anti α_{2u}-globulin antibody

α_{2u}-globulin as an antigen was obtained from the urine collected from aged male rats, pooled, and used to immunize rabbits. The immunization procedures, including the amount of antigen and immunizing intervals, were determined from the results of a preliminary test referring to the methods of Kurtz *et al.* (1976). The antigen was injected under the skin at a dose of 1 mg/animal (1st injection) or 0.5 mg/animal (2nd and subsequent injections) once at two weeks. Blood sampling was conducted periodically and the antibody titer measured. When the antibody titer level reached a plateau, whole blood was collected and antiserum was obtained from the blood. The antiserum was used for immunohistochemistry and immuno-electron microscopy. For measurement of the α_{2u}-globulin content in the urine and tissues, the antibody was purified from the antiserum using a DEAE ionic exchange column after ammonium sulfate precipitation. The singularity of the antibody was confirmed as a single diffuse band of approximately 19 kDa by Western blotting analysis. This study and the following study were carried out in accordance with the Law for the Humane Treatment and Management of Animals and the Standards Relating to the Care and Management, etc. of Experimental Animals in Japan.

Experiment 1 Confirmation of specific reactivity of the antibody to α_{2u}-globulin

1. Preparation of α_{2u}-globulin nephropathy rats

To confirm the specific reactivity of the anti-α_{2u}-globulin antibody, we prepared α_{2u}-globulin nephropathy rats as follows. Male and female Crj:CD(SD)IGS rats were obtained from Charles River Japan Inc. and used at the age of 11 weeks. d-Limonene (Nacalai Tesque Inc.), a well-known α_{2u}-globulin nephropathy inducer, was administered to the rats, consisting of 4 males and 4 females each, for 10 days at doses of 0, 150 and 300 mg/kg/day by gavage using corn oil as a vehicle. The rats were housed individually in stainless steel wire cages in an animal room with a controlled temperature of 24±2°C, humidity of 55±10% and a 12-hr light/dark cycle (lighting from 7:00 to 19:00) and allowed access to food and water ad libitum.

Pooled urine was collected for 24 hr on the day before the start of administration and on Day 9 of administration. After the 10-day administration period,

the rats were anesthetized with intraperitoneal injection of 30 mg/kg of sodium pentobarbital and perfused with physiological saline-added lactose (Lactec, Otsuka Pharmaceutical Factory Inc.) through the sinus aortae, after which the liver and kidneys were removed. The urine and a part of the liver and kidneys were used for measurement of their α_{2u}-globulin content and the remainder of the liver and kidneys for histopathology, immunohistochemistry and immuno-electron microscopy. The samples for histopathology and immunohistochemistry were embedded in paraffin following fixation with 10% neutral buffered formalin solution for about two weeks. The samples for immuno-electron microscopy were dehydrated with an ascending series of ethanol and embedded in spurr resin following pre- and post-fixation with 2.5% glutaraldehide and 1% osmium tetroxide solutions, respectively.

2. Histopathology and immunohistochemistry

The serial paraffin sections were prepared, deparaffinized and then stained with hematoxylin and eosin (HE) accompanied by Azan-Mallory staining and periodic acid shiff(PAS) reaction.

For immunohistochemistry, the paraffin sections were deparaffinized and incubated with 0.25% pronase E for 20 min at 37°C, after which they were washed 3 times in Tween-PBS (PBS containing 0.1% Tween 20, pH7.6). The specimens were incubated with 0.3% H_2O_2 in methanol at room temperature for 30 min to inactivate the endogenous peroxidase activity, and then washed 3 times in Tween-PBS. After blocking against nonspecific immuno-reactions with 10% FCA was conducted at room temperature for 20 min, the sections were incubated overnight with rabbit anti-α_{2u}-globulin antiserum at 4°C at a dilution of 1:80000 in PBS containing 1% BSA. Negative controls were incubated with an equivalent volume of diluent solution alone. The sections were washed 3 times in Tween-PBS and incubated with biotynilated secondary antibody (goat anti-rabbit and goat anti-mouse immunoglobulins, Dako, LSAB2 kit) at room temperature for 30 min. After they were washed 3 times inTween-PBS, the sections were incubated with horseradish peroxidase (HRP)-labelled streptavidin (Dako, LSAB2 kit) at room temperature for 30 min. The sections were then washed 3 times in PBS and reacted with 3,3-diaminobenzidine (DAB) for 5 min. The reactions were quenched by placement in running tap water, and the sections were then counterstained lightly with methylgreen, dehydrated in n-butanol, cleaned in xylene, and mounted.

3. Immuno-electron microscopy

Ultra-thin sections were prepared and reacted overnight with the anti-α_{2u}-globulin antiserum at a dilution of 1:5000 at 4°C. Protein A-colloidal Gold (10 nm, British Bio Cell International Inc.) was used at a dilution of 1:10, after which the sections were double stained with uranyl acetate and lead citrate.

4. Measurement of α_{2u}-globulin content in the liver, kidneys and urine

The α_{2u}-globulin content was measured in the liver and kidneys in all males in all the groups of α_{2u}-globulin nephropathy rats, and in the urine in two males each in the control and highest dose groups. The liver and kidneys were homogenized with phosphate buffer weighing 4 times their tissue weights and centrifuged at 105,000 g for one hour. The protein content of the supernatant thus obtained was measured for every molecular weight and the urine was measured similarly as is. Western blotting was then conducted using purified anti-α_{2u}-globulin antibody and the content of the protein showing a positive reaction was regarded as α_{2u}-globulin content.

Experiment 2 α_{2u}-globulin analysis for industrial chemicals

The selected chemicals are listed in Table 1. We selected 10 chemicals, which are suspected of being CIGA, among all the chemicals in the Japanese Existing Chemicals Survey Program (JECSP). In addition, two chemicals which caused renal toxicity without hyaline droplet accumulation were selected as negative controls. We used paraffin-embedded renal specimens originating from the JECSP toxicity studies conducted in several laboratories and stored for four to seven years in each. For each toxicity study, three groups (the control and low- and high-dose groups for 11 chemicals) or two groups (the control and high-dose groups for the other) were selected. The low-dose group has the dose showing the lowest effect for hyaline droplets in tubules or other renal changes, and the high-dose group has the highest dose administered in each toxicity study. The doses selected for each chemical are described in Table 1. Three male specimens were arbitrarily selected for each dose group based on the results obtained from HE-stained sections in the original studies.

The serial paraffin sections were prepared, deparaffinized and then stained with HE accompanied by Azan-Mallory staining and PAS reaction. The sections were also stained immunohistochemically using anti-

Table 1. Chemical name and effect dose derived from the general toxicity studies.

Chemical	Test type	Original study doses (mg/kg/day)	Effect doses (mg/kg/day) [a]			Original reported NOEL (mg/kg/day) [a]	The selected doses for analyzing (contr./low/high) (mg/kg/day)
			Histopathological findings		Non histopathological observations		
			AN	Other			
1,4-Dibromobenzene	RD	0/ 4/ 20/100/500	20≤ / –	100≤	100≤ / 20≤	4	0/ 20/500
Dicyclopentadiene	RT	0/ 4/ 20/100	4≤ / –	20≤ / 100	20≤ / 100	<4 / 20	0/ 4/100
3,4-Dimethylaniline	RD	0/10/ 50/250	50≤ / –	250	250 / 50≤	10	0/ 50/250
1,4-Dicyanobenzene	RD	0/ 1.25/ 5/ 20/ 80	5≤ / –	20≤ / –	20≤	1.25 / 5	0/ 5/ 80
Tetrahydrothiophene-1,1-dioxide	RD	0/60/ 200/700	200≤ / –	–	700	60 / 200	0/200/700
1,3-Dicyanobenzene	RD	0/ 8/ 40/200	8≤ / –	40≤ / 200	40≤	<8 / 8	0/ 8/200
Acenaphthene	RD	0/12/ 60/300	60≤ / –	300	300 / 60≤	12	0/ 60/300
3,4-Dichloro-1-butene	RT	0/ 0.4/ 2/ 10/ 50	10≤ / –	50	10≤ / 50	2 / 10	0/ 10/ 50
3a,4,7,7a-Tetrahydro-1*H*-indene	RT	0/ 67/200/600	67≤ / –	600	67≤ / 200≤	<67 / 67	0/ 67/600
3,5,5-Trimethylhexan-1-ol	RT	0/ 12/ 60/300	12≤ / –	60≤	60≤	12	0/ 12/300
2,4-di-*tert*-butylphenol	RD	0/ 5/ 20/ 75/300	– / –	300	300 / 75≤	75 / 20	0/ – /300
4-aminophenol	RD	0/ 4/ 20/100/500	– / –	100≤	100≤	20	0/100/500

[a] The data were described in a pattern of male/female when the data were different between the male and female.
RD, 28-day Repeat Dose Toxicity Test; RT, Combined Repeat Dose and Reproductive/Developmental Toxicity Test.
AN, α_{2u}-globulin nephropathy including hyaline droplets and subsequent tubular alteration.

α_{2u}-globulin antiserum by the above-mentioned protocol. HE-stained sections were used to examine the degree of hyaline droplets and to determine whether or not other findings were present. The degree of occurrence of hyaline droplets was divided into five grades, including none (−), minimal (±, barely detectable minimal appearance), slight (+, multifocal but not dispersed appearance), moderate (++, dispersed appearance over the cortex) and severe (+++, diffused appearance over the whole cortex). The staining sections with PAS, Azan-Mallory and anti-α_{2u}-globulin reaction were also graded similarly for positive-stained droplets. In addition, computational image analysis was carried out to verify the above-mentioned grading criteria using three typical immuno-stained samples for each grade. Images including almost all the renal superficial cortex were captured using a light microscope (Olympus BHS) and a digital camera (Olympus DP12). The captured images were measured for positive area using an image analyzing system (C-Imaging System, Compix Inc.), and the positive area (%) was then calculated from the data.

RESULTS

Experiment 1 Specific reactivity of the antibody to α_{2u}-globulin

On the HE-stained sections of the kidneys, hyaline droplets with round to irregular shapes were observed in the renal proximal tubular epithelium only in males administered d-limonene (Photo. 1a). The hyaline droplets were negative for PAS reaction (Photo 1b) but stained positively with Azan-Mallory staining (Photo 1c). With immuno-staining with the anti-α_{2u}-globulin antibody, the hyaline droplets were more clearly stained and more distinguishable than with Azan-Mallory staining (Photo 1d). The hyaline droplets showed a dose-dependent increase on the HE-stained sections (Photo 2, a-c) and positive reactions for hyaline droplets showed a correlational increase with immuno-staining (Photo 2, d-f). Very fine positive granules were also detected on the immuno-stained sections for all the males as background, but no positive reactions were observed in other tissue components. This background was observed generally in male kidneys and was, therefore, excluded from the grading in experiment 2. In the liver, all the males showed a positive reaction for the antibody in centrilobular hepatocytes. The degree of intensity was weaker than in the kidneys, and there was no clear intensification by d-limonene. No positive reaction for the anti-α_{2u}-globulin antibody was detected in the liver or kidneys in any females.

With electron microscopy, electron-dense and irregular-shaped inclusions surrounded by a single membrane were observed as changes corresponding to the hyaline droplets in the renal proximal tubular epithelium, and positive reactions were observed for the antibody with post-embedding method in the inclusions (Photo 3). A similar positive reaction was observed in the lysosomes of the renal tubule epithelium, but no positive reaction was detected in the hepatocytes.

The α_{2u}-globulin content in the kidneys of the males was increased dose-dependently by administration with d-limonene (Fig. 1). A dose-dependent but mild increase in α_{2u}-globulin content was also observed in the liver of the males. While no dose-dependent increase in the urine was noticeable, a lower molecular type of α_{2u}-globulin appeared in the males in the highest dose group, with the α_{2u}-globulin type reported as an early marker for α_{2u}-globulin nephropathy (Saito *et al.* 1991).

Experiment 2 α_{2u}-globulin analysis for industrial chemicals

Table 2 indicates the grades of all the samples with respect to hyaline droplets, positive droplets and immunological positive droplets analyzed with HE, Azan-Mallory and anti-α_{2u}-globulin antibody staining, respectively. In the controls there was a minimal to moderate amount of hyaline droplets in some animals and consequent variation for Azan-Mallory and anti-α_{2u} -globulin reaction. This variation was due to the arbitrary sampling of specimens, or probably related to the lot of the animals or to the difference of food used in each study. Dose-dependent increases of hyaline droplets in the renal proximal tubular epithelium were, however, confirmed for HE-staining of 10 chemicals suspected of being CIGA (1,4-dibromobenzene, dicyclopentadiene, 3,4-dimethylaniline, 1,4-dicyanobenzene, tetrahydrothiophene-1,1-dioxide, 1,3-dicyanobenzene, acenaphthene, 3,4-dichloro-1-butene, 3a,4,7,7a-tetrahydro-1H-indene, 3,5,5-trimethylhexan-1-ol). This was described in the original reports (Toxicity Testing Reports of Industrial Chemicals), although the occurrence of hyaline droplets varied in shape, size and number/cell with chemicals and showed no clear common features. In the highest dose groups of these chemicals, basophilic tubules, granular casts in the tubules and/or tubular dilatation were intensified or occurred as in the original reports. These changes

showed similar features in spite of the various severity and incidence with the chemicals. In serial sections prepared simultaneously, Azan-Mallory-positive reactions for hyaline droplets were detected dose-dependently in these 10 chemicals. No PAS-positive reaction was detected in any chemical. These staining behaviors of the hyaline droplets were the same as those in the case of d-limonen described above. Immunohistochemical staining using the anti-α_{2u}-globulin antibody revealed thoroughly dose-dependent positive reactions for hyaline droplets in all these chemicals. The resulting grades from three types of analysis were the same, demonstrating that a highly positive correlation exists among the three staining methods. As for the remainder not suspected of being CIGA (2,4-di-tert-butylphenol, 4-aminophenol), there was no increase of hyaline droplets or positive immunohistochemical reactions in any dose groups, as well as no stain in either PAS or Azan-Mallory staining. In addition, computational image analysis using three typical immuno-stained sections for each grade (Photo 4) showed a close correlation between the quantitative analysis and semi-quantitative grading (Fig. 2).

DISCUSSION

Many toxicity studies using laboratory animals have been conducted on environmental and industrial chemicals to ensure their safety or toxicity levels concerning human health. On extrapolating the results to humans, toxic mechanisms that are unlikely to occur in humans should be taken into account. A typical example of such toxicities is α_{2u}-globulin-related nephropathy and the consequent renal tumorigenesis in repeated

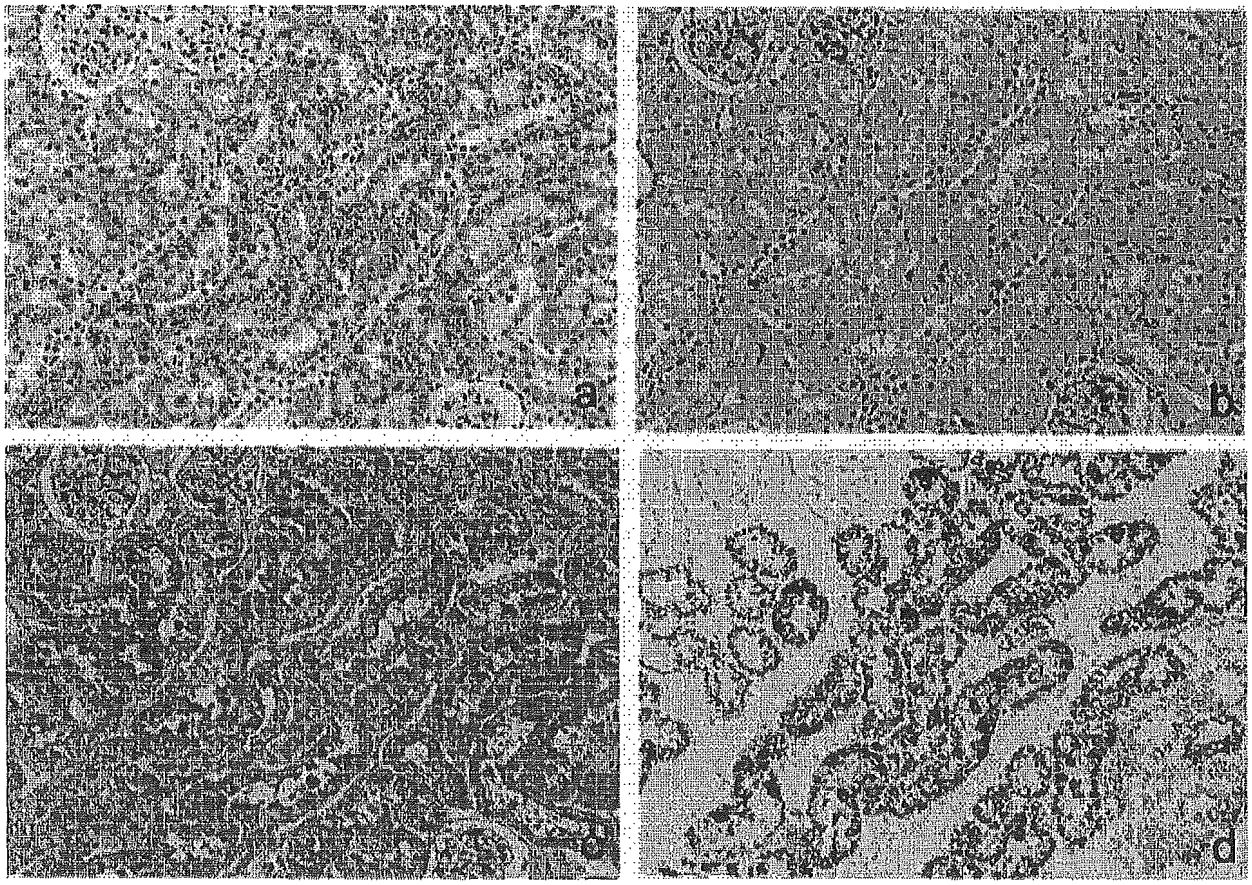

Photo 1. d-Limonene induced hyaline droplet accumulation in the kidney (HE, a). The hyaline droplets were PAS-negative(b), but they were stained positively with Azan-Mallory staining (c). Immunohistochemistry using the anti-α_{2u}-globulin antibody showed a clear positive reaction consistent with the hyaline droplets (d). Original magnification, ×66.

dose toxicity studies using male rats. This male rat-specific nephrotoxicity is not considered to occur in humans (Hard *et al.*, 1993). To exclude this male rat-specific toxicity from chemical risk assessment, it is necessary to demonstrate properly that such renal toxicity results from α_{2u}-globulin-CIGA complex accumulation. Detection analysis of α_{2u}-globulin in the nephrotoxicity has not been conducted in most conventional toxicity studies, however, especially in sub-acute toxicity screening studies for industrial chemicals. As

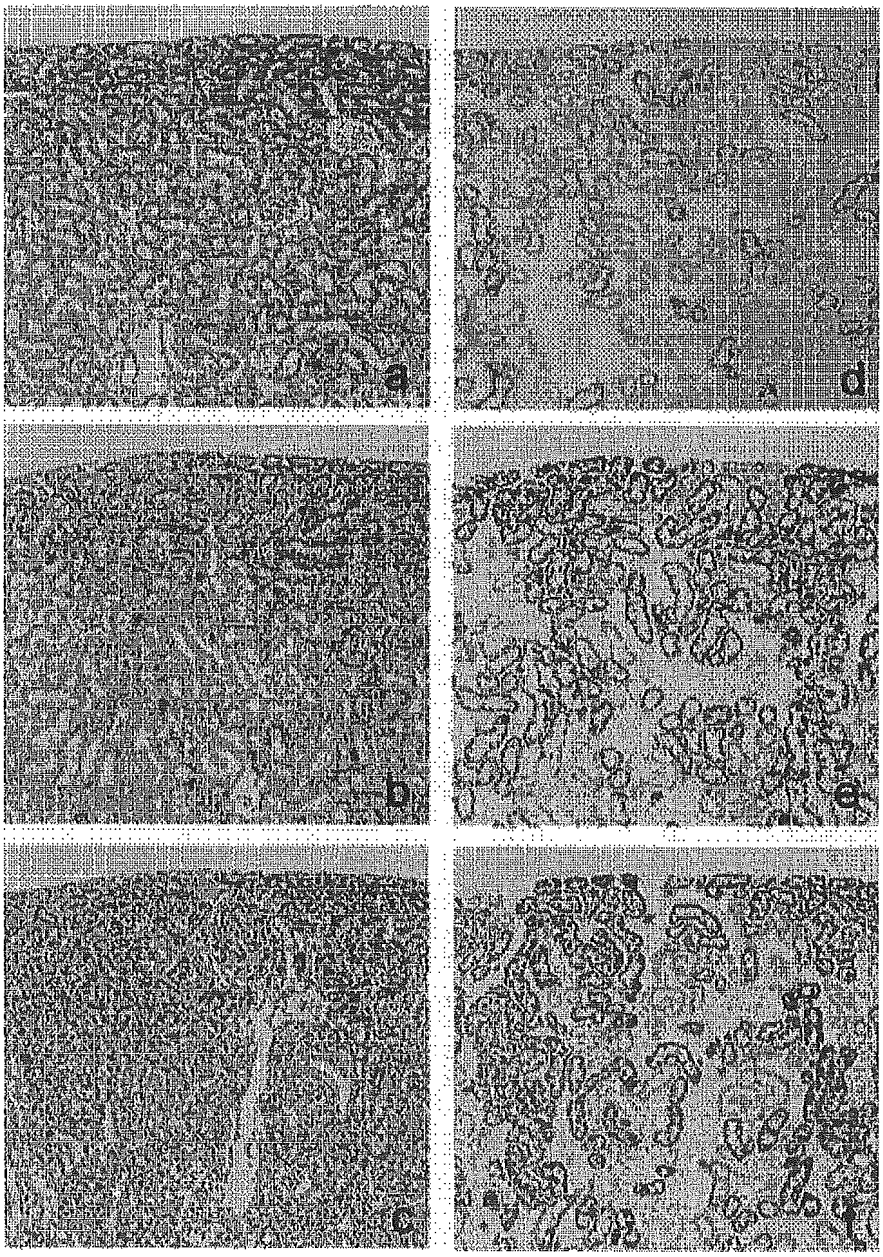

Photo 2. An increase of hyaline droplets in the kidney in correlation to the doses of *d*-limonene(HE, a - c). Positive reaction for the anti-α_{2u}-globulin antibody also increased with similar dose dependency (d - f). Original magnification, ×33.

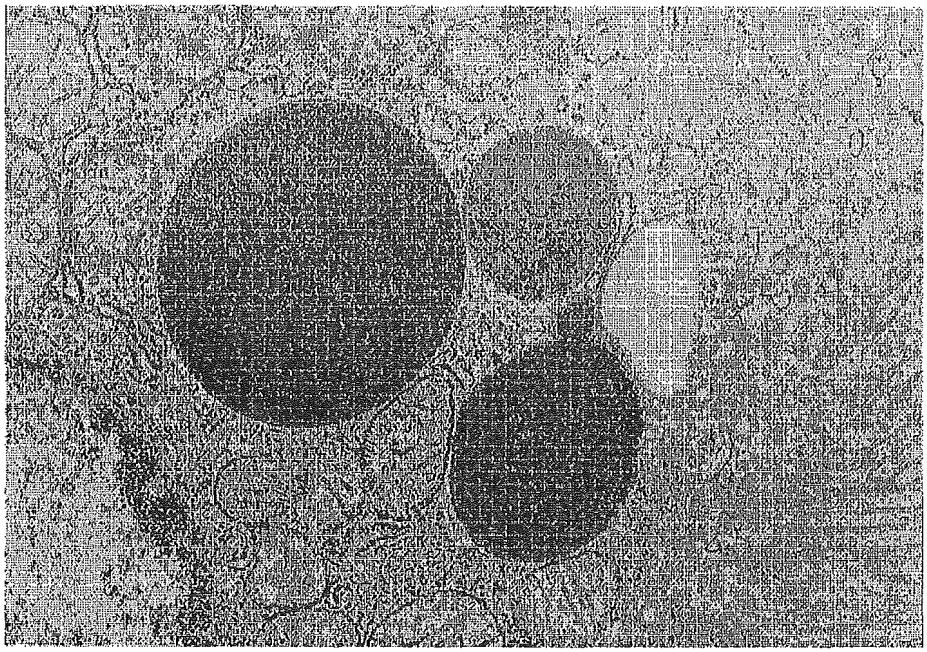

Photo 3. Immuno-electron micrograph of cytoplasmic inclusions, corresponding to the *d*-limonene induced hyaline droplets, in the epithelial cell of the renal proximal tubule. Colloidal gold particles are dispersed in the inclusions. Original magnification, ×10,000.

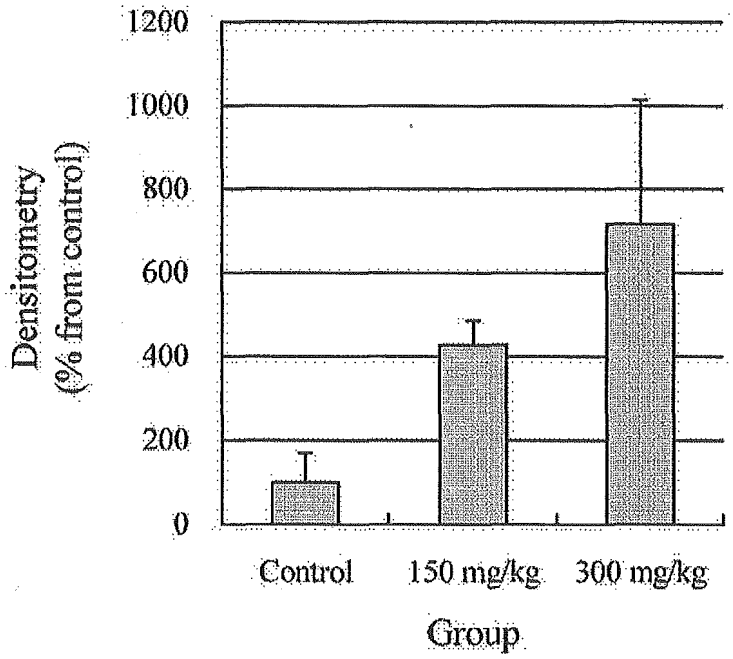

Fig. 1. Western blot analysis of α_{2u}-globulin in kidney from male rats treated with *d*-limonene.
Results are expressed as mean ± SD (n=4).

an alternative detection method, it is well known that α_{2u}-globulin droplets in the kidneys are negative for PAS reaction, but that they are stained positively by Azan-Mallory staining (U.S. EPA, 1991; Alden *et al.*, 1984). Although these additional stainings can distinguish hyaline droplets resulting from α_{2u}-globulin accumulation from those resulting from other causes, these analyses provide only indirect evidence. Direct evidence of α_{2u}-globulin accumulation in renal hyaline droplets could be required for appropriate risk assess-

Table 2. Grading results of histological/histochemical examination.

Chemical	Staining	Results		
		Control	Low dose	High dose
1,4-Dibromobenzene	HE [1]	–/–/±	++/++/+	++/+++/+++
	Azan-Mallory [2]	–/–/±	++/++/+	++/+++/+++
	Anti-α_{2u}-globulin [2]	–/–/±	++/++/+	++/+++/+++
Dicyclopentadiene	HE	–/–/–	+/++/++	+++/+++/+++
	Azan-Mallory	–/–/–	+/++/++	+++/+++/+++
	Anti-α_{2u}-globulin	–/–/–	+/++/++	+++/+++/+++
3,4-Dimethylaniline	HE	–/–/–	–/–/±	±/±/+
	Azan-Mallory	–/–/–	–/–/±	±/±/+
	Anti-α_{2u}-globulin	–/–/–	–/–/±	±/±/+
1,4-Dicyanobenzene	HE	–/–/–	±/+/+	++/+++/+++
	Azan-Mallory	–/–/–	±/++/+	+++/+++/+++
	Anti-α_{2u}-globulin	–/–/–	±/++/+	+++/+++/+++
Tetrahydrothiophene-1,1-dioxide	HE	+/–/–	+/+/++	++/++/++
	Azan-Mallory	+/–/–	++/+/++	++/++/++
	Anti-α_{2u}-globulin	+/–/–	++/+/++	++/++/++
1,3-Dicyanobenzene	HE	–/–/±	+/±/±	++/++/+++
	Azan-Mallory	–/±/±	+/±/±	++/+++/+++
	Anti-α_{2u}-globulin	–/±/±	+/±/±	++/+++/+++
Acenaphthene	HE	±/–/+	+/–/+	+/+/++
	Azan-Mallory	±/–/+	+/±/+	+/+/++
	Anti-α_{2u}-globulin	±/–/+	+/±/+	+/+/++
3,4-Dichloro-1-butene	HE	–/–/++	+/+/±	++/+/++
	Azan-Mallory	–/–/++	+/+/+	++/+/++
	Anti-α_{2u}-globulin	–/–/++	+/+/+	++/+/++
3a,4,7,7a-Tetrahydro-1*H*-indene	HE	+/+/++	++/++/++	+++/+++/+++
	Azan-Mallory	+/+/++	++/++/++	+++/+++/+++
	Anti-α_{2u}-globulin	+/+/++	++/++/++	+++/+++/+++
3,5,5-Trimethylhexan-1-ol	HE	–/–/±	+/+/++	+++/++/+++
	Azan-Mallory	±/–/±	+/+/++	+++/++/+++
	Anti-α_{2u}-globulin	±/–/±	+/+/++	+++/+++/+++
2,4-Di-tert-butylphenol	HE	–/–/–		–/–/–
	Azan-Mallory	–/–/–		–/–/–
	Anti-α_{2u}-globulin	–/–/–		–/–/–
4-Aminophenol	HE	–/±/–	–/–/–	–/–/–
	Azan-Mallory	–/±/–	–/–/–	–/–/–
	Anti-α_{2u}-globulin	–/±/–	–/–/–	–/–/–

[1] Grading for hyaline droplets.
[2] Grading for positive droplets.
No PAS-positive reaction for the hyaline droplets was observed in any sample.
Low dose for 2,4-di-*tert*-butylphenol was not examined.