在することが明らかとなった[5]。また，nSP70が細胞質内に侵入すると，活性酸素種（ROS）が産生され，それに起因すると考えられるDNA障害が生じ得ることを明らかとしている[6]。

また，nSP70の体内移行後のハザード情報を，静脈内に過剰量のnSP70を投与することにより収集した。その結果，nSP70が血中移行後，肝臓から胎盤・胎仔に至るまで，全身臓器に分布し得ることが明らかとなった。また，分布した組織において，①肝障害が誘発されること，②胎盤傷害に起因すると考えられる，胎仔吸収率の増加や，胎仔体重の減少等が誘発されること，等が明らかとなった[7]。上記の検討はあくまでも過剰量の静脈内投与によるハザード評価であるため，今後，実際の曝露量を加味した検討や経皮塗布による検討が必須ではあるが，以上の結果から，ナノサイズの非晶質シリカは従来のバルクサイズの非晶質シリカとは異なる体内動態を示すと共に，種々のハザードが増大する可能性が示された。

次に，こうして得られたハザード情報を指標に，安全なナノマテリアルに関する情報の収集に向けて，ナノマテリアルの表面修飾がその安全性に及ぼす影響を評価した。nSP70の表面がカルボキシル基，アミノ基で修飾されたnSP70-C，nSP70-Nのハザード情報を収集した。その結果，nSP70で認められたヒト皮膚角化細胞のROS産生，マウスの肝障害や胎仔吸収率の増加，胎仔体重の減少等が一切認められず，ハザードが減弱することを見出した[7]。我々は既に，nSP70の表面修飾により，その細胞内動態が変わること[9]，nSP70により誘発される免疫毒性や急性毒性等のハザードをも軽減可能であることを明らかとしており，表面修飾がナノマテリアルの安全性確保における優れたアプローチであることを認めている。

以上，ナノマテリアルは従来までのバルクサイズの素材とは異なる体内動態・生体影響を示し，新たなハザード／リスクが生じ得ること，そうした体内動態や生体影響はナノマテリアルの表面修飾等の物性を制御することにより調整可能であることを明らかとした。ナノマテリアルの中にも安全性が高いものとそうでないものがあることはよく知られているが，今後，安全なナノマテリアルを創製するための方法論といったNano-Safety Scienceに関する情報をより多く収集することが，ナノマテリアルの安全性研究の最重要課題の一つであると考えている。なお，現状において実用化されているナノマテリアルには各種の修飾が施されていると考えられるが，上記の知見から，実用化されているナノマテリアルの多くは安全性が高いと考察している。

このように，試薬グレードの多様なナノマテリアルを用いた検討により，有効かつ安全なナノマテリアルに関する基礎情報が収集できつつある。一方で，より詳細にナノマテリアルの安全性を評価するためには，実サンプルを用いた安全性評価が必須であることは言うまでもなく，我々は，企業からサンプルの提供を受け，それらの安全性を鋭意評価しているところである。本稿では紙面の都合上，我々の知見の一例のみ紹介させて頂いたが，今後，こういったNano-Safety Science研究を積み重ねることで，ナノマテリアルを応用した，安心・安全な香粧品製品等の開発支援に尽くしていきたいと考えており，我々は安全性研究に加え，その有効活用研究にも積極的に取り組んでいる。

4　おわりに

本総説では，実験用グレードの非晶質シリカを用いた検討を中心として，ナノマテリアルが発揮する，従来までのバルクサイズの素材とは異なる体内動態や生体影響に関する情報の一部を紹介した。また，安全性の高いナノマテリアル開発に資する基盤情報として，ナノマテリアルの表面修飾を最適化することで，ナノマテリアルのハザードが減弱し，安全かつ有用なナノマテリアルを開発できることを示した。この安全なナノを開発できるという事実は，最も重要な知見であり，類を見ない，安全かつ有用なナノを我が国が開発し，他を圧倒する知財を確保できることを示しており，今後の進むべき道しるべと言えよう。なお，今回は深く取り上げなかったが，動物愛護の観点から，ナノマテリアルの安全性評価においても，香粧品分野でも多用されるヒト細胞株活性化試験（h-CLAT）等，動物実験に替わる代替法の確立，利用が重要となってくると考えられる。我々は，今後，

Nano-Safety Science研究を更に推し進め，ナノマテリアルの物性と生体影響との連関を追及すると共に，表面修飾の制御によってハザードを減弱できるメカニズムを明らかとすることで，安全なナノマテリアルの創製に資する有用な情報を得られるものと考えている。そうして得られた知見が広く公表され，産業応用されることが，有用かつ安全なナノマテリアル製品の開発，引いてはナノマテリアル産業界の更なる発展に繋がると期待している。

謝辞

本研究は，厚生労働科学研究費補助金化学物質リスク研究事業等の支援を受けて実施されたものです。この場をお借りして深謝申し上げます。

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Quantifying the biodistribution of nanoparticles

To the Editor — Yamashita *et al.* (*Nature Nanotech.* **6,** 321–328; 2011) report that silica and titanium dioxide (TiO_2) nanoparticles with diameters of 70 nm and 35 nm, respectively, can cross the placental barrier in pregnant mice. Using transmission electron microscopy (TEM), the researchers claim that nanoparticles are found in the liver and brain of the fetus[1]. Although TEM is useful for the qualitative examination of nanoparticles, it is not sensitive enough for studying the trans-placental transport of TiO_2 nanoparticles.

Assuming that the concentration of TiO_2 nanoparticles in the fetal liver is one nanogram per gram of liver (the density of liver is approximately 1.1 g cm^{-3}) and that the mass of a 35 nm TiO_2 particle is approximately 1×10^{-16} g (the density of rutile-TiO_2 is 4.3 g cm^{-3}), on average, only one nanoparticle can theoretically be found in a 1 mm^2 section of liver tissue (an ultrathin section usually has a thickness of less than 100 nm). The TEM images collected by Yamashita *et al.* showed a dark electron-dense spot in a field size of ~5 μm × 5 μm. Based on our estimation, on average, tens of thousands of such images need to be examined to find one TiO_2 nanoparticle. This means that the TEM results cannot firmly prove that nanoparticles were present in the fetal liver and brain, unless the concentration of nanoparticles in the fetal liver is several orders of magnitude higher than the hypothetical value of one nanogram per gram.

In conclusion, more suitable quantitative methods should be used to study the biodistribution of nanoparticles in pregnant mice. ❐

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To the Editor — In general, transmission electron microscopy (TEM) and quantitative methods such as inductively coupled plasma-mass spectrometry (ICP-MS) are used to study the biodistribution of nanomaterials. For example, ICP-MS can detect the elements of nanomaterials and evaluate their biodistribution quantitatively. However, ICP-MS cannot distinguish between elements that are inherent to the nanomaterials and those that are cleaved or released from them. But, unlike ICP-MS, TEM can detect the presence of nanomaterials and identify their location within tissues and cells. Even though the TEM images in our study[1] provide only qualitative information, TEM is invaluable for identifying the biodistribution of the silica and TiO_2 nanoparticles.

He *et al.*[2] correctly point out the detection limit of TEM and that only some silica and TiO_2 nanoparticles could be detected in the small section of the placental and fetal tissue in our study. However, through analysis of several hundreds of TEM sections, we confirmed that silica and TiO_2 nanoparticles did accumulate in both the placental and fetal tissues. The observations were not coincidental.

To study the biodistribution of nanomaterials quantitatively, methods such as ICP-MS should be used and, indeed quantitative biodistribution studies of silica and TiO_2 nanoparticles in the mouse placenta and fetus are currently under way in our laboratory. ❐

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—Review—

ナノマテリアルの細胞内移行性とROS産生/DNA損傷との連関解析

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Relation Analysis between Intracellular Distribution of Nanomateriarls, ROS Generation and DNA Damage

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(Received August 31, 2011)

With recent development of nanotechnology, nanomaterials (NMs) have been developed with innovative function and expected to cause a paradigm shift in various industry such as cosmetics, medicine and food. NMs begin to establish firm position in Japan as base of various industrials, in fact, a part of them have been already applied to various products. On the other hand, it is suggested that these innovative properties may induce unknown biological responses. It is concerned about the effect of these innovative properties to human health. Based on these situations, to evaluate risk of NMs, it is started to collect information about safety of NMs (Nano Safety Science). With this in mind, we analyzed the relationship between particle size and the *in vitro* effect of amorphous nanosilica (nSP) using human keratinocyte cells (HaCaT). Our results indicate that exposure to nSP of 70 nm diameter (nSP70) induced an elevated level of reactive oxygen species (ROS), leading to DNA damage. On the other hand, a markedly reduced response was observed using submicron-sized silica particles. Next, we investigate relationship between endocytosis, generation of ROS and DNA damage using endocytosis inhibitor, cytochalasin D (CytoD). As result, CytoD -treatment reduced nSP70-mediated ROS generation and DNA damage. This suggested that endocytosis is involved in nSP70-mediated cellular effects. Thus, particle size affects amorphous silica-induced ROS generation and DNA damage in HaCaT cells. We believe that clarification of the endocytosis pathway of nSP will provide useful information for hazard identification as well as the design of safer forms of nSP.

Key words——nanomaterial; Nano Safety Science; amorphous nanosilica; ROS generation; DNA damage

1. はじめに

近年のナノテクノロジーの発展に伴い，少なくとも1次元の大きさが100 nm以下の素材，いわゆるナノマテリアルの開発が進んでいる．ナノマテリアルは，従来までのサブミクロンサイズ以上（100 nm以上）の素材とは異なり，サイズの微小化により組織浸透・拡散能などの革新的な機能を有することが明らかとなっており，香粧品，医薬品，食品分野などの種々産業にパラダイムシフトを起こす新素材になるものと期待されている．ナノマテリアルは既に，知財立国を目指すわが国において各種産業を支える基盤としての確固たる地位を築き始めており，事実，その一部が配合された製品が既に実用化・上市されている．その一方で，このようなナノマテリアル特有の物性に起因する革新的機能が逆に，ヒトの健康環境に負の影響，いわゆるナノ毒性（NanoTox）を及ぼす可能性が懸念され始めている．[1,2] 例えば，カーボンナノチューブが，マウスモデルにおいてアスベストと同様に悪性中皮腫発症の危険性を有すること，[3,4] ナノ酸化チタンを経鼻曝露したマウス脳内で酸化ストレス応答が誘発されるこ

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本総説は，日本薬学会第131年会シンポジウムGS03で発表したものを中心に記述したものである．

となど.[5] 各種ナノマテリアルのハザード情報が続々と報告されている．こうした中，ナノマテリアルの安全性評価に向けて，経済協力開発機構（OECD）や国際標準化機構（ISO）などの国際機関が中心となって，ナノマテリアルのリスク評価に必要な安全性に関する基礎情報の収集，いわゆるナノ安全科学研究（Nano Safety Science 研究）が世界規模で推進されている．わが国においても，経済産業省や厚生労働省，環境省によってナノマテリアルの安全性情報の収集とその手法の開発が進められており，粒径 100 nm 以下のナノマテリアルが従来までのサブミクロンサイズ以上の素材とは異なる体内吸収性や体内動態を示すことが，少しずつ判明してきている．筆者らの検討結果においても，直径が 100 nm 以下の非晶質ナノシリカが，①皮膚角質バリアを通過して体内に吸収され得る可能性，[6] ②妊娠マウスを用いた静脈内投与のモデルにおいて，胎盤を介して胎児の肝臓や脳に移行する可能性を見い出している．[7] また，体内に移行し組織に分布したナノマテリアルは，最終的に組織細胞に取り込まれる可能性が考えられる．事実，筆者らのグループでは *in vitro* のモデルを用いた検討において，直径がサブミクロンサイズ以上の非晶質シリカと 100 nm 以下の非晶質ナノシリカの細胞内局在を比較したところ，異なる局在を示すことを明らかとしている．[6,8] しかし，ナノマテリアルの細胞内局在を考慮した細胞応答の評価など，安全性評価に向けた情報は世界的にもいまだ不足している．

ナノマテリアルは，化粧品材料や食品添加物を始めとして，既に様々な分野でわれわれヒトに直接適用される製品に使用されている．この事実は，年齢や疾患の有無を問わず，あらゆるヒトが一生涯に渡ってナノマテリアル含有製品を使用・摂取し得ることを示唆しており，たとえわずかな量であっても，長期に渡って曝露することによって健康に問題が生じる可能性が危惧される．したがって，ナノマテリアルの使用が増加しつつある今こそ，ナノマテリアルの安全確保，安全なナノマテリアルの開発を目指したナノ安全科学研究を推進せねばならない．しかしながら現状は，いまだ断片的なハザード情報が散在するのみで，安全性評価指針の策定や安全なナノマテリアルの設計指針につながる情報の抽出には至っておらず，ナノマテリアルの細胞毒性など現象論のみが先行しており，詳細なメカニズムに関する検討は圧倒的に不足しているのが現状である．したがって，ヒトの健康を確保しつつナノマテリアルの恩恵を最大限に享受した豊かな社会の実現するためには，ナノマテリアルと従来までのサブミクロンサイズ以上の素材との体内/細胞内動態や生体/細胞への影響を比較検討し，相違点及びメカニズムを科学的根拠に基づいて明らかにすることが急務である．そして，それらの情報を基盤として，より具体的なナノマテリアルの安全性評価法を確立することが最重要課題である．以上の観点から筆者らは，種々のナノマテリアルの中でも，生産量・使用量・用途の点で，最もわれわれの生活に浸透している非晶質ナノシリカをモデルナノマテリアルとして，素材の物性（サイズ，表面電荷，親/疎水バランス，形状など）が体内/細胞内動態や生体/細胞応答に与える影響の解析を進めている．[6-12] これらナノマテリアルの物性–動態–安全性の連関情報を集積し，それらの因果関係を精査することによって，最終的にナノマテリアルの動態情報から安全性を評価する方法の確立，及び安全なナノマテリアルの開発と実用化の支援の実現につながるものと考えている．本稿では，非晶質ナノシリカが多くの香粧品に既に使用されているという現状を踏まえて，皮膚細胞への影響を中心に検討を進め，特に，様々なサイズの非晶質シリカについて，細胞内局在や細胞への影響を解析した結果を紹介する．

2. 非晶質ナノシリカによる DNA 損傷と ROS 産生の連関解析

非晶質ナノシリカは，数あるナノマテリアルの中でも極めて使用量が多く，国内生産量は約 20000 トン，世界での年間生産量は 1 メガトン以上であり，その市場規模は 3 億 1400 万ドルにも及ぶ．[13] 非晶質ナノシリカは，非常に幅広い産業分野で実用化されており，例えば，日焼け止めやファンデーションなどの化粧品基剤材，歯磨き粉や歯の充填剤，食品の固結防止剤に使用されている．[13] 一方で安全性に関しては，2006 年の欧州化学物質生態毒性・毒性センターの報告によると，従来までのサブミクロンサイズ以上のシリカに問題はないとされているが，ナノシリカについての情報はほとんど皆無に等しい．つまり，ナノメートルサイズの非晶質シリカは適用範囲・生産量・ヒトの曝露機会が圧倒的に高い

素材であるにもかかわらず，安全性が未知のまま使用されており，数あるナノマテリアルの中で最も安全性評価が急がれている素材である．筆者らはこれまでの検討において，分散性の高い直径 70 nm の非晶質ナノシリカ（nSP70）が皮膚バリアを通過し肝臓にまで移行する可能性を示している．[6] また，*in vitro* のモデルを用いた検討においても，直径がサブミクロンサイズ以上の非晶質シリカは細胞質内にのみ局在が認められたのに対し，100 nm 以下の非晶質ナノシリカは細胞質のみならず，核内にまで移行していることが明らかとしている．[5,8] 以上の結果より，非晶質シリカは，粒子径の違いによってその細胞内局在が大きく変動することが明らかとなった．したがって，香粧品素材として既に使用され始めている非晶質ナノシリカをモデル粒子として用い，皮膚細胞を対象とした直径 100 nm 以下のナノシリカの細胞内における詳細な局在情報/細胞応答の連関情報を集積することは，ナノ安全科学研究において極めて重要であると言える．特に，nSP70 が核内にまで移行するという特徴的な細胞内動態を示すことを加味すると，核機能や遺伝子に着目した安全性評価を行うことが必要であることが強く示された．

そこで本検討では，非晶質ナノシリカの細胞内における局在情報/細胞応答について，ヒト皮膚角化細胞株（HaCaT 細胞）を用いて主に核機能や遺伝子に着目した検討を実施した．まず，コメットアッセイにより非晶質シリカの HaCaT 細胞における DNA 損傷性を評価した．nSP70, nSP300, mSP1000 を 30, 90 μg/mL の濃度で添加し，3 時間後に Tail length の値を指標に評価したところ，nSP300 と mSP1000 を添加した群では DNA 損傷は全く検出されなかった．一方，nSP70 添加群においては，陽性コントロールとして使用した過酸化水素添加群に匹敵するほどの DNA 損傷性の増大が認められた．この結果から，100 nm 以下のサイズの nSP70 のみが，HaCaT 細胞に対して DNA 損傷を引き起こすことが示された．そこで次に，非晶質ナノシリカによる安全性確保を目指して，活性酸素種（Reactive oxygen species: ROS）産生の観点から HaCaT 細胞の DNA 損傷の発現メカニズムの解明を試みた．まず，HaCaT 細胞を用いて非晶質シリカ処理による ROS 産生の有無を検証した．nSP70, nSP300, mSP1000 を HaCaT 細胞に添加して 3 時間後の細胞内における ROS 量を DCFH-DA の蛍光量を指標に測定した．その結果，すべてのサイズの非晶質シリカを添加した群において ROS の産生が認められたが，特に nSP70 添加群において最も顕著な ROS 産生が認められた．コメットアッセイで用いたシリカと同じ濃度で，ROS 産生が起こっていることが確認されたことを踏まえると，直径が 100 nm 以下になると ROS 依存的な DNA 損傷作用を発揮する可能性が示唆された．そこで，nSP70 による ROS 産生と DNA 損傷発現メカニズムとの関連について精査するために，抗酸化剤である N-アセチルシステイン（NAC）存在下における nSP70 の DNA 損傷作用を評価した．NAC を前処理した HaCaT 細胞に対して，30 分後に nSP70 を添加し，コメットアッセイを行った．粒子添加 3 時間後に ROS 産生量を定量したところ，NAC を前処理した群では，PBS 添加群と同等の値にまで Tail Length の値の減少が認められた．抗酸化剤共存下で nSP70 依存的 DNA 損傷作用の抑制が認められたことから，nSP70 による DNA 損傷が ROS を介して生ずることが裏付けられた．すなわち，この事実を言い換えると，nSP70 の ROS 産生や細胞内局在を制御することで，安全なナノマテリアルの創製が実現するものと考えられた．

3. 非晶質ナノシリカの細胞内移行性と DNA 損傷/ROS 産生の連関解析

近年，細胞の ROS 産生経路の 1 つとして NADPH oxidase に注目が集まっている．[14] NADPH oxidase は異物が細胞内に取り込まれる際にエンドソーム膜にリクルートされ，ROS 産生を通じて異物の除去に寄与することが知られている．例えば，アスベストや結晶性シリカなどの微粒子状の異物が炎症を引き起こす際に，粒子が細胞に侵入することにより発現する NADPH oxidase の関与が報告され，これらの報告では NADPH oxidase から産生された ROS が NALP3 を活性化することが明らかとされている．[15-17] これらの知見から，nSP70 の細胞内移行後の ROS 産生や DNA 損傷に，NADPH oxidase が関与している可能性が考えられた．そこでまず，NADPH oxidase は，粒子が細胞に取り込まれる際にそのエンドソーム膜にリクルートされ，ROS 産生を通じて異物の除去に寄与することをふまえ，

nSP70 の ROS 産生/DNA 損傷発現メカニズムに対して細胞内移行が関与しているかについて精査した．アクチン重合を阻害して細胞の貪食能を阻害するサイトカラシン D (CytoD) を用いて，非晶質ナノシリカによる DNA 損傷性及び ROS 産生に対する細胞内移行の関与を検証した．あらかじめ播種しておいた HaCaT 細胞に CytoD を処置し，その後 CytoD 共存下で nSP70 を添加した．SP70 を添加した 3 時間後，ROS 産生は DCFH-DA の蛍光量を指標に測定し，DNA 損傷性はコメットアッセイを用いて解析した．その結果，CytoD を前処置することで，nSP70 による ROS 産生は PBS 添加群と同等の値にまで抑制され，さらに DNA 損傷性についても同様の傾向が認められた．以上の事実から，nSP70 による ROS 産生や DNA 損傷には，細胞内移行が必須であり，細胞内に移行した後の反応をより詳細に解析する必要性が示された．そこで次に，NADPH oxidase 阻害剤である Apocynin を用いて，NADPH oxidase と nSP70 による ROS 産生や DNA 損傷発現メカニズムとの関連を精査した．あらかじめ播種しておいた HaCaT 細胞に Apocynin を処置し，その後 Apocynin 共存下で nSP70 を添加した．nSP70 を添加した 3 時間後，ROS 産生は DCFH-DA の蛍光量を指標に測定し，DNA 損傷性はコメットアッセイを用いて解析した．その結果，Apocynin を前処置した HaCaT 細胞においては，nSP70 に起因した ROS 産生の減弱が認められた．一方で，nSP70 による DNA 損傷性には有意な変化は認められなかった．以上の事実から，nSP70 による DNA 損傷の発現には NADPH oxidase の関与も一部あるとは考えられるものの，別の機構が強く関与していることが示された．これらの点を踏まえて，現在筆者らは DNA 損傷発現メカニズムに関して，以下の 3 つの観点から解析を進めている (Fig. 1)．1 つ目として，nSP70 によって産生された ROS がシグナル応答を誘導し，最終的に DNA 損傷を引き起こす経路である．ROS はこれまで，Akt/P13K や MAP K シグナルなど複数のシグナル応答に関与していることが報告されており，nSP70 の細胞内移行経路とそれぞれの応答を精査する必要がある．2 つ目は，nSP70 が細胞内に移行した後にミトコンドリアなどの細胞内小器官に移行し損傷を与えることにより，細胞内に ROS が産生され，その結果 DNA 損傷が引き起こされる経路である．特にミトコンドリアは ATP 産生の際に ROS を産生し，自身で産生した ROS が起因となりミトコンドリア自身の DNA 損傷が誘導されていることが知られている．[18] この点からも，nSP70 の細胞内局在と細胞内

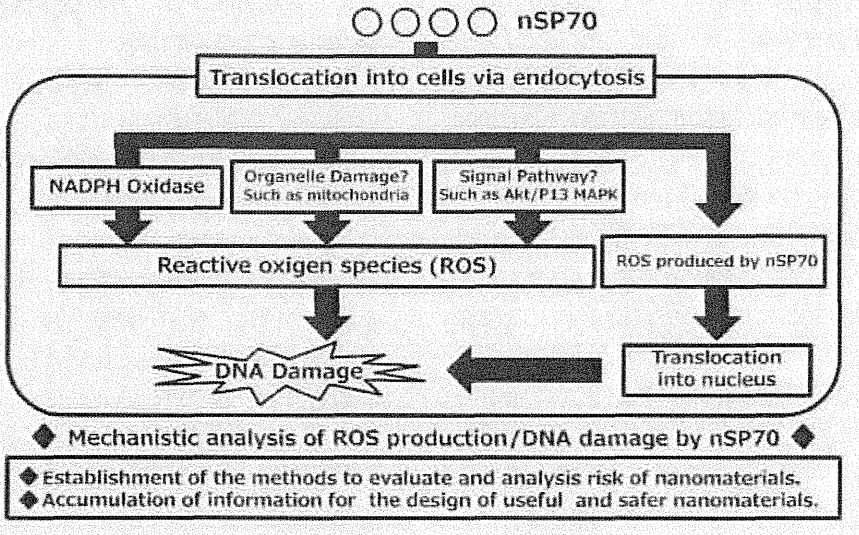

Fig. 1. Hypothesis of nSP70-mediated ROS Production Mechanism

Our results indicate that exposure to nSP70 induced an elevated level of ROS, leading to DNA damage. A markedly reduced response was observed using submicron-sized silica particles of 300 and 1000 nm diameter. In addition, cytochalasin D-treatment reduced nSP70-mediated ROS generation and DNA damage, suggesting that endocytosis is involved in nSP70-mediated cellular effects. Thus, particle size and internalization route, and physicochemical properties affect nSP70-induced ROS generation and DNA damage of HaCaT cells. We believe clarification of the endocytosis pathway of nSP will provide useful information for hazard assessment as well as the design of safer forms of nSPs.

小器官に与える影響を解析する必要がある．3つ目は，nSP70自身がROS産生能を有しており，nSP70が核内に移行することによりDNA損傷が引き起こされる経路である．ナノ酸化チタンなどは粒子自身がROSを産生し，抗菌作用やDNA損傷を発揮することが知られており[19]，筆者らも予備的な結果ではあるが，nSP70自身がROSを産生する可能性を見い出している．この点からも，nSP70の細胞内局在と細胞内小器官に与える影響を解析する必要がある．今後，それぞれ，細胞内移行経路，細胞内局在，核内移行経路といった細胞内局在の詳細な解析を通じて，DNA損傷発現/ROS産生メカニズムを明らかにする予定である．

4. おわりに

本研究では，最もわれわれの生活に浸透している非晶質ナノシリカの細胞内移行とDNA損傷発現/ROS産生について連関解析を行った．その結果，nSP70によるDNA損傷の誘導やROS産生には，細胞内移行が必須であり，また，nSP70によるDNA損傷発現には，NADPH oxidase以外の別の機構が関与していることが示された．これらの結果は，非晶質ナノシリカの安全性評価を行う際には，第一に従来型シリカとは別個の素材として捉え独自の安全性評価を行う必要があることを裏付けている．また，今回はDNA損傷発現/ROS産生メカニズムに着目して解析を進めたが，現在，核内移行性という情報に基づいたプロテオミクス解析により，ナノシリカの曝露によって発現変動する核内タンパク質を多数見い出している（未発表データ）．これら発現変動タンパク質と核機能との連関解析によって，細胞内移行と細胞応答の因果関係をより精査できるものと考えている．また，細胞内局在のみならず生体内動態と安全性の連関解析を行うとともに，表面物性（表面電荷など）との連関解析も進行している．既に筆者らは，表面物性を制御することで，非晶質ナノシリカによる生殖発生毒性を回避できる可能性[7]やDNA損傷発現に関しても同様に抑制できることを見い出している（未発表データ）．筆者らのナノ安全科学研究を基盤として，非晶質ナノシリカのみならず，あらゆるナノマテリアルに応用可能な安全性評価法を確立することで，科学的根拠に基づいた安全なナノマテリアルの使用・設計指針の策定が実現するものと期待している．

謝辞　本研究は，日本学術振興会科学研究費補助金基盤研究B一般（No. 21390046），日本学術振興会科学研究費補助金（学術研究助成基金助成金）挑戦的萌芽研究（No. 23659078），文部科学省地域科学技術振興施策知的クラスター創成事業，厚生労働科学研究費補助金化学物質リスク研究事業（No. H19-化学一般-005及びH22-化学一般-006），厚生労働科学研究費補助金子ども家庭総合研究事業斉藤班（H20-子ども一般-002），環境省地球環境研究総合推進費，内閣府食品安全委員会食品健康影響評価技術研究（No. 1005），財団法人コスメトロジー研究振興財団，財団法人喫煙科学研究財団，財団法人武田科学振興財団の支援を賜りました．ここに深謝申し上げます．また，本総説で紹介した研究内容は，大阪大学大学院毒性学分野 堤 康央教授（創薬基盤研究所バイオ創薬プロジェクト チーフプロジェクトリーダーを併任）の統括の下，日本化粧品工業連合会，大阪大学臨床医工学融合研究教育センター 特任准教授 吉岡靖雄先生，大阪大学大学院薬学研究科 助教 吉川友章先生，特任助教 鍋師裕美先生，医薬基盤研究所バイオ創薬プロジェクトプロジェクトリーダー 角田慎一先生，サブプロジェクトリーダー 鎌田春彦先生，プロジェクト研究員 阿部康弘先生，長野一也先生を始めとする多くの方々の連携によって得られた共同成果であり，この場をお借りして御礼を申し上げます．

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Materials **2012**, *5*, 350-363; doi:10.3390/ma5020350

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Review

Carbon Nanomaterials: Efficacy and Safety for Nanomedicine

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Received: 16 December 2011; in revised form: 11 February 2012 / Accepted: 15 February 2012 / Published: 21 February 2012

Abstract: Carbon nanomaterials, including fullerenes, carbon nanohorns, and carbon nanotubes, are increasingly being used in various fields owing to these materials' unique, size-dependent functions and physicochemical properties. Recently, because of their high variability and stability, carbon nanomaterials have been explored as a novel tool for the delivery of therapeutic molecules including peptide and nucleic acid cancer drugs. However, insufficient information is available regarding the safety of carbon nanomaterials for human health, even though such information is vital for the development of safe and effective nanomedicine technologies. In this review, we discuss currently available information regarding the safety of carbon nanomaterials in nanomedicine applications, including information obtained from our own studies; and we discuss types of carbon nanomaterials that demonstrate particular promise for safe nanomedicine technologies.

Keywords: nanomedicine; carbon nanomaterials; fullerenes; carbon nanohorns; carbon nanotubes; drug delivery; nano safety science

1. Introduction

Advances in nanotechnology have led to the recent development of many nanomaterials, including nanoscale silica particles, titanium dioxide nanoparticles, and carbon nanomaterials [1–4]. Nanomaterials, which are generally classified as materials with feature sizes smaller than 100 nm, have remarkably impacted various fields of study because of the desirable properties (e.g., enhanced electrical conductivity, tensile strength, and chemical reactivity) imparted by their increased surface area per unit weight compared with that of their bulk-scale counterparts [5,6]. Nanomaterials are already being applied in electronics [1], foods [2], and cosmetics [3]. Furthermore, in basic research for development of new drugs, nanomaterials are expected to open novel avenues for the treatment of human diseases owing to their unique physicochemical properties [4].

Carbon nanomaterials, including fullerenes, carbon nanohorns (CNHs), and carbon nanotubes (CNTs), have been used as carriers in drug delivery and other applications [7]. Carbon nanomaterials with carbon cage and graphene structures have many technological advantages such as facile modification by functional groups [8–10], high carrier capacity [11,12], high chemical stability [13,14], and feasibility of incorporating both hydrophilic and hydrophobic substances [15,16] (Table 1). These characteristics, which are essential for the development of drug-delivery carriers, make carbon nanomaterials promising for nanomedicine applications.

Table 1. Basic physicality of carbon nanomaterials.

	Fullerenes	**CNHs**	**CNTs**
Year of discovery	1985	1998	1991
Discoverer	H.W. Kroto R.F. Curl R.E. Smalley	S. Iijima	S. Iijima
Size			
Diameter	1 nm	2–4 nm	0.4–70 nm
Length	-	40–70 nm	1 μm–2.5 mm
Shape	sphere	horn	fiber
Practical use	Cosmetics Lubricity agent Semiconductor	Fuel battery	Semiconductor Car parts Sports goods

Though highly promising, these carbon nanomaterials are in an early phase of development. Therefore, information regarding their safety is not sufficient for the development of medically sound and nontoxic technologies. Because nanomaterials' physicochemical properties often differ substantially from those of their bulk counterparts, as mentioned above, there are concerns that carbon nanomaterials may exhibit unexpected side effects. In addition, recent reports have shown that pristine CNTs might induce mesothelioma-like lesions in mice, similar to those induced by asbestos [17–19]. On the other

hand, Muller *et al.* showed that pristine CNTs induce no mesothelioma formation in a 2-year *in vivo* study [20]. Therefore, more information about the safety of nanomaterials needs to be collected.

In this review, we discuss currently available information about the safety of carbon nanomaterials for nanomedicine applications, including information obtained from our own previous studies. We also discuss types of carbon nanomaterials that demonstrate particular promise for safe nanomedicine technologies.

2. Utility of Carbon Nanomaterials for Nanomedicine

2.1. Fullerenes

Fullerenes have attracted considerable attention in various fields of science [21]. Fullerenes are composed entirely of carbon in the form of a hollow sphere, ellipsoid, or tube. Spherical fullerenes are also referred to as buckyballs. An important property of the fullerene molecule is their high symmetry. There are 120 symmetry operations, such as rotation around an axis and reflection in a plane. Fullerenes belong to the class of inorganic nanoparticles and show high bioavailability due to their small size (~1 nm). Owing to their small size, fullerenes can penetrate various tissues and organelles that materials with submicron size cannot penetrate. For example, Foley *et al.* reported that fullerenes can cross the COS-7 cell membrane and bind to the mitochondria [22], demonstrating that fullerenes have utility as intracellular carriers. Furthermore, fullerenes' capability to act as drug-delivery carriers for low-molecular-weight compounds and oligonucleotides has been demonstrated [23]. For example, conjugates composed of fullerenes and paclitaxel have exhibited the potential to provide slow release of the drug and have exhibited significant anti-cancer activity in cell cultures [24]. Moreover, Maeda-Mamiya *et al.* reported effective gene delivery *in vivo* using water-soluble fullerenes [25]. In that study, conjugates consisting of cationic tetraamino fullerenes and an insulin-gene-expressing plasmid complex were injected intravenously into C57/BL6 mice. Insulin gene expression was detected in the lung, liver, and spleen. Plasma insulin levels in the insulin gene group of mice were significantly higher than those in a control group. Both of these studies demonstrate that fullerenes may act as drug- and gene-delivery carriers. Furthermore, because fullerenes are strong anti-oxidants, they have been used as neuroprotective [26,27] and anti-inflammatory agents [28]. Thus, if fullerenes can be controllably manipulated, they could be used to treat various diseases.

2.2. CNHs/CNTs

CNHs and CNTs based on the structure of graphene are also regarded as drug-delivery carriers [29,30]. CNHs and CNTs are differentiated from each other by their shape and size (Table 1). Furthermore, CNHs and CNTs can be classified as possessing either single- or multi-walled (SW or MW) structures. This review cites several reports on the use of SWCNHs and SWCNTs as drug-delivery carriers. SWCNHs have plenty of inner spaces. Through these holes, various molecules such as low-molecular-weight compounds or nucleic acids can enter the hollow interior of the SWCNHs. SWCNHs also can regulate the sustained release of drugs from their interior for drug-delivery applications. For example, the release rate of cisplatin (CDDP), a chemotherapy drug that can be incorporated into oxidized SWCNHs, has been regulated by controlling solvent composition. The release of CDDP from the SWCNHs is slower in water and a culture medium than in

phosphate-buffered saline, and the CDDP released from SWCNHs in the former solvent effectively kills human lung-cancer cells [11].

SWCNTs also have been demonstrated to be amenable for drug delivery. SWCNT-siRNA conjugates have been efficiently transported to human T-cells and primary cells, which are inert to commercially available liposome-based nonviral vectors, and have silenced a specific gene in those cells [31]. In cancer therapy, SWCNT-based tumor-targeted drug-delivery system (DDS) has already been developed by several investigators [32,33]. SWCNT-anticancer-drug conjugates also have shown higher efficacy in suppressing tumor growth than clinical anticancer drugs alone in various cancer models [32,33]. These therapeutic effects were induced by accumulation of the conjugates in tumor. Collectively, these results clearly indicate the potential applications of SWCNHs and SWCNTs in cancer-targeted drug delivery and sustained release [34].

2.3. Suitable Modification of Carbon Nanomaterials for DDS

Accumulation at a targeted location is important in DDS. Carbon-nanomedicine-based cancer treatment systems generally function by means of either active targeting or passive targeting. In active-targeting DDS for cancer treatment, the search for cancer-specific targets is important. SWCNTs modified with antibodies, folate, arginine-glycine-aspartic acid (rgd) peptide, and epidermal growth factors have been useful for active targeting of tumor tissue [35–38]. Ruggiero *et al.* reported that antibody-modified SWCNTs accumulate in tumor tissue in a murine xenograft model of human colon adenocarcinoma [39]. However, these anti-cancer effects are not enough to enable drug development because these targets do not express specifically in tumor. In recent years, novel targets have been identified by using "-omics" approaches such as proteomics, genomics, and metabolomics [40–42]. Proteomics-based analysis is currently a promising approach for identifying biomarker proteins for use in drug development because these proteins directly regulate the onset and progression of diseases. However, proteomics-based analysis can yield many potential candidate biomarker proteins that are over- or under-expressed in diseased tissues, and these candidates must be efficiently screened to identify appropriate targets. Toward this end, we have developed an "antibody proteomics system" that facilitates the screening of biomarker proteins from many candidates by rapid preparation of cross-reacting antibodies using phage antibody library technology. The system is an efficient method for screening tumor-related biomarker proteins to identify novel targets [43].

In passive-targeting DDS for cancer treatment, improvement of drug retention in blood is important because the reticulo-endothelial system and kidney work as the barrier against foreign particles *in vivo*. Covalent conjugation of polyethylene glycol (PEG) to a carrier's surface, referred to as "PEGylation" is a promising strategy to improve retention of various nanomaterials in the blood [44]. PEGylation can prolong the plasma half-life and alter the tissue distribution of the nanomaterial conjugates compared with their non-PEGylated forms, which typically clear the body through the reticulo-endothelial system *in vivo*. The extended circulating lifetime of PEGylated conjugates in blood induces an enhanced permeability and retention effect, which is based on the leaky nature of tumor blood vessels, resulting in increased delivery of the conjugates to tumor tissue. As an example, Yang *et al.* investigated the long-term *in vivo* biodistribution of nanoscale graphene sheets functionalized with PEG and systematically examined the potential toxicity of graphene over time [45]. On the other hand, from the

aspect of effectivity and safety, CNTs kinetics is important for drug development. Singh *et al.* describes the pharmacokinetic parameters of intravenous administered functionalized SWCNTs relevant for various therapeutic and diagnostic applications [46]. It shows that functionalized (water-soluble) SWCNTs can, in fact, be excreted via the renal route. In summary, to obtain highly effective and nontoxic carbon nanomaterial DDS for cancer treatment, it is necessary to control three factors: (1) size; (2) the ability to target the molecules to tumors and (3) clearance through the reticulo-endothelial system and kidney.

2.4. Other Application of CNTs in Medicine

As mentioned above, CNTs have been explored as a novel tool for the delivery of therapeutic molecules including peptide, nucleic acid and cancer drugs. On the other hand, certain types of CNTs have been reported to possibly help cancer diagnosis and other application [47,48]. Photoacoustic imaging proposes higher spatial resolution and permits deeper tissues to be imaged compared with most optical imaging techniques. Zerda *et al.* [47] showed plain SWCNTs conjugated with cyclic Arg-Gly-Asp (RGD) peptides can be used as a agent for photoacoustic imaging of tumors. This report indicates SWCNTs is possibly useful for cancer diagnosis. Additionally, Tosun *et al.* suggested collagen conjugated SWCNTs show the potential for enhanced electrical activity. These SWCNTs have been shown positive *in vitro* biocompatibility results offering further evidence that SWCNT-based materials have an important role in neuronal regeneration [48]. Neurodegenerative disorders including Parkinson's and Alzheimer's diseases, amyotrophic lateral sclerosis are rapidly increasing as the population ages. The field of nanomedicine promises revolutionary advances to the diagnosis and treatment of devastating human diseases [48].

3. Safety of Carbon Nanomaterials

3.1. Hazard Assessment

Carbon nanomaterials are among the most promising nanomedicines. However, information about the safety of carbon nanomaterials is still fragmentary, and ensuring their safety is of utmost importance to protect human health. In this section, we focus on the safety of CNTs specifically, because some studies have reported that CNTs have higher toxicity than fullerenes and CNHs [49].

Parameters such as structure, size distribution, surface area, surface chemistry, surface charge, and agglomeration state as well as purity of the samples, have considerable impact on the reactivity of CNTs. Some studies have reported that certain types of SW or MW CNTs are cytotoxic and genotoxic *in vitro*, so public concern about the potential risk of CNTs to human health has risen [50–52]. In fact, recent reports have indicated that certain types of CNTs might induce mesothelioma-like lesions in mice, in a manner similar to that observed for mesothelioma induced by asbestos [53–55]. Takagi *et al.* showed that intraperitoneally administered pristine MWCNTs induce mesothelioma in the p53 (+/−) mouse carcinogenesis model, probably due to the MWCNTs' resemblance to asbestos in size and shape and to their biopersistency [17]. Poland *et al.* also observed asbestos-like pathogenic behavior of long pristine MWCNTs associated with their needle-like fiber shape and established a structure-activity relationship based on the length of the MWCNTs [19]. These studies revealed that the propensity of

long MWCNT fibers to produce inflammation and fibrosis in the peritoneal cavity is similar to, or greater than, that of long asbestos fibers. In contrast, neither short asbestos fibers nor short tangled MWCNTs cause any significant inflammation [19]. These results suggest that physical properties, such as length, diameter and physico-chemical properties, might impact the safety of pristine CNTs [56]. However, these studies were based on the administration of extremely high doses of MWCNTs via peritoneal injection. In contrast, Shvedova *et al.* showed that pristine MWCNTs enhanced acute inflammation and pulmonary injury with delayed bacterial clearance after aspiration or inhalation of MWCNTs [57,58]. In the future, it is needed to examine the study relevant to the human occupational exposure situation.

There are a few reports that examine the mechanisms of CNT toxicity [59–61]. One important underlying factor that influences the safety of long fibers is the failure of macrophage cells to completely enclose them. This failure, termed incomplete or "frustrated" phagocytosis, can induce inflammation [19]. Migliore *et al.* showed that long rigid MWCNTs appear to form fiber-like aggregates or structures that are too long to be phagocytosed by macrophage cells, thus resulting in reactive oxygen species (ROS) production [62,63], which contributes to NACHT domain-, leucine-rich repeat-, and pyrin domain-containing protein 3 (NLRP3) activation [64,65]. Palomaki *et al.* demonstrated that the NLRP3 inflammasome was essential for long, needle-like CNTs and asbestos to induce IL-1β secretion [65]. Moreover, it was noted that CNT-induced NLRP3 inflammasome activation depended on ROS production [65]. Clarification of the mechanism of inflammation induced by CNTs might lead to the development of safe carbon nanomedicine technologies.

Although some studies have reported concern about the safety of CNTs as mentioned above, other studies have reported that certain types of CNTs are safe materials for nanomedicine. Yang *et al.* demonstrated that after intravascular injection of pristine SWCNTs, mice did not show stress or symptoms of abnormality, such as lethargy, anorexia, or changes in body weight [66]. Furthermore, Wick *et al.* showed that the cytotoxicity of purified rope-like agglomerated SWCNTs was lower than that of well-dispersed SWCNTs [67]. In addition to the fiber-like structure of CNTs, the amount of metal contaminants such as iron or nickel found in the CNTs may contribute to the nanomaterials' potential carcinogenicity by accelerating the generation of ROS [68–70]. Moreover, in preparation for drug development, it is important to examine the influence of the oxidative debris on CNTs [14,71–73]. It is an emergent and key point during purification and functionalization of carbon nanostructure. These studies suggest that the safety of CNTs is determined not only by physical properties but also by a wide variety of factors such as method of administration, dispersability, and presence of metal contaminants. How much these factors contribute to the safety of CNTs remains unknown, however. We believe that the information obtained by these safety studies might be useful for ensuring the safety of CNTs.

3.2. Biological Behavior of CNTs

Evaluation of *in vivo* kinetics is important for assessing the safety of nanomedicine technologies. In this section, studies about the behavior of CNTs in the body are described. Ruggiero *et al.* showed that intravascularly injected pristine SWCNTs favor liver accumulation and hepatobiliary excretion over kidney accumulation and renal excretion [39]. In addition, several studies have investigated pulmonary

effects subsequent to instillation, aspiration, and inhalation of pristine SWCNTs [57]. These reports showed that short and small tangles of SWCNTs that deposit subpleurally migrate to the pleural space and exit in the flow of pleural fluid through the stomata, where they follow the lymphatic drainage to the mediastinal lymph nodes [60,74]. In addition, long carbon nanotubes also reach the pleural space but cannot negotiate the stomata, and so they are retained in the pleural space, where they cause inflammation and potentially long-term disease [60,74].

Kagan *et al.* showed that hypochlorite and reactive radical intermediates of the human neutrophil enzyme myeloperoxidase catalyze the biodegradation of carboxylated SWCNTs *in vitro*, in neutrophils and to a lesser degree in macrophages [75]. Importantly, the biodegraded nanotubes do not generate an inflammatory response when aspirated into the lungs of mice [75]. In addition, Liu *et al.* have reported that the biodurability of SWCNTs depends on surface functionalization [76]. Based on these findings, strategies for mitigating the pro-inflammatory effects of these nanomaterials in occupational settings may be developed.

Furthermore, information about toxicokinetics (absorption, distribution, metabolism and elimination) also should be obtained for the development of safe CNTs.

3.3. Development of Safe Nanomaterials

We have discussed above how nanomaterials can serve as useful nanomedicine technologies and have also highlighted the importance of considering these nanomaterials' safety for such applications. In this section, we examine the current status of the development of safe and effective nanomaterials for nanomedicine. In our own studies, we have established relationships between the physical properties and safety of CNTs. Our data showed that pristine thin MWCNTs and SWCNTs do not induce genetic damage *in vitro* and inflammation *in vivo* [77]. These data indicate that physical properties such as particle length and width might influence the safety of CNTs. In addition, Nagai *et al.* suggested the large-diameter or tangled MWCNTs are less toxic, less inflammogenic, and less carcinogenic than untangled MWCNTs [56]. These results suggest that control of the diameter of CNTs could be used to develop CNTs that are safe for human health.

Furthermore, in addition to being critically important for the detection of biomolecules, the surface properties of nanomaterials also can modulate the materials' safety. Recent studies have shown that functionalization of CNTs with carboxyl or amino surface groups can affect the CNTs' toxicity [78]. Thus, regulation both of particle size and of surface properties is considered important for research leading to the development of safe nanomedicine technologies.

In fact, our previous study showed that nanoscale silica particles, which we expected to be useful as drug-delivery carriers, display different intracellular localization compared with submicron- and micro-scale silica particles and induce a greater cytotoxic response to mouse macrophage cell line [79]. We have also shown that nanoscale silica particles induce certain cellular responses, such as ROS generation and DNA damage to human keratinocyte cell line [80]. These results indicate that particle size could influence the silica particles' safety for applications in nanomedicine. In addition, we have shown that surface modification of silica particles with functional groups, such as amino or carboxyl groups, suppresses toxic biological effects of silica particles including inflammatory responses and ROS production [81]. A recent study demonstrated that nanomaterials become coated with serum

proteins and induce different cellular responses from intact particles by binding to proteins [82]. In addition, different surface characteristics, such as surface charge, influence the binding affinities of proteins to nanomaterials [82,83]. In fact, Gasser *et al.* showed that functionalization of MWCNTs have the potential to alter the MWCNTs blood plasma protein coating in biological systems [84,85]. These results indicate that particle size or surface properties of carbon nanomaterials can affect their safety, and that control of these physical properties can be used to advance the development of safe nanomaterials.

4. Conclusions

The unique physicochemical properties of carbon nanomaterials allow them to incorporate targeting ligands, chemotherapeutic drugs, and many other therapeutic agents that have great potential for cancer-targeted therapy. However, owing to the large number of factors that influence the kinetics of drug release from nanomaterials, as well as their safety for human health, insufficient information is available on these two important subjects. Factors that influence the safety of and kinetics of drug release from nanomaterials include their shape, length, and dispersability, as well as the presence of metal contaminants. A detailed understanding of the pharmacological and toxicological properties of carbon nanomaterials, as well as a balanced evaluation of their risks and benefits to human health, is required before they can be recommended for routine clinical use. We believe that a detailed safety analysis of carbon nanomaterials will be invaluable for the design of safe nanomedicine technologies.

Acknowledgements

This study was supported in part by Grants-in-Aid for Scientific Research from JSPS; by Health Labour Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan; by a Global Environment Research Fund from Minister of the Environment; by Food Safety Commission; by The Cosmetology Research Foundation; by The Smoking Research Foundation; by The Takeda Science Foundation.

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