になった。続いて、nSP70 投与マウスを用いて典型的な DIC 症状である消費性凝固障害（血小板数減少、血液凝固遅延など）や血管透過性の亢進の有無を評価した（図 2）。その結果、直径 300 nm 以上の nSP を投与したマウスにおいては消費性凝固障害が認められなかったのに対して、nSP70 投与マウスでは血中血小板数の著しい減少や血液凝固時間の有意な延長が認められた。さらに、エバンスブルーの血管外漏出を指標に nSP70 投与マウスの血管透過性を評価したところ、100 nm 以下の nSP を投与したマウスにおいてのみ、四肢・耳・鼻の劇的な青変が認められた（図 3）。先述した通り、ガラスやコラーゲンと言った異物表面は、血液凝固第 XII 因子との接触により、その立体構造を変化させることで活性化を補助することが知られており、nSP70 の血中への侵入によって第 XII 因子の活性化を起点とする血液凝固カスケードが促進された可能性が考えられた。そこでこの点を解析するために、各 SP の精製ヒト第 XII 因子活性化能を評価した（図 4）。いずれの SP を適用した場合においても第 XII 因子の活性化が認められたもの、その効果は nSP70 適用群において最も強かった。以上の結果は、nSP70 投与による急性致死毒性が DIC 様症状の誘発によるものであることを示唆すること、これらの現象が 100 nm 以下の nSP で最も強く活性化されうる可能性を示している。現在、nSP の比表面積と血液凝固活性化との相関を調べると共に、nSP と血液凝固因子との結合性の解析を進めている。

続いて、血液凝固系の活性化と nSP の表面物性との連関を追求した。まず、表面修飾ナノシリカの急性毒性を評価した（図 5）。その結果、表面未修飾の nSP70 投与マウスは図 1 と同様に投与後 12 時間以内に死亡した。その一方で、nSP70-C あるいは nSP70-N を投与したマウスにおいては急性致死毒性が全く認められなかった。さらに、これらのマウスの PT や APTT を測定したところ、nSP70-C あるいは nSP70-N において血液凝固の活性化が緩和されることが明らかとなった。以上の結果から、nSP70 の粒子表面をカルボキシル基やアミノ基で修飾することで血液凝固系の活性化を緩和でき、それを反映して急性致死毒性をも回避できることが明らかとなった（図 6）。表面修飾体の急性致死毒性回避効果が、粒子表面と第 XII 因子との相互作用の低減によるものであると考え、各 nSP の第 XII 因子活性化能を解析した（図 7）。その結果、nSP70 適用群と比較して nSP70-N 適用群の第 XII 因子の活性化は有意に減弱していた。しかし nSP70-C 適用群においては、予想に反して nSP70 適用群と同程度に第 XII 因子が活性化されることが明らかとなった。すなわち、nSP70-C の急性致死毒性回避効果には、第 XII 因子との相互作用以外の要因が関与している可能性が考えられた。一般に、第 XI 因子、プレカリクレイン、高分子キニノゲンといった第 XII 因子以外の血液凝固因子は異物表面との接触を介して活性化されることが知られている。今回は、第 XII 因子活性化能のみを対象として解析したが、今後はその他の凝固因子の活性化についても nSP の表面物性との関連を精査する必要があると思われる。また、研究分担者の角田・八木らが明らかにしているように、nSP70 の血中への侵入が急性肝組織障害を誘発することにも留意する必要があろう。傷害された組織からは、一般に組織因子（Tissue factor）と呼ばれる血液凝固因子が発現することが知られている。つまり、nSP70 によって傷害された肝組織から組織因子が発現した可能性が強く示唆されるため、これが起点となって血液凝固が促進されたものと考えることもできる。現在、①粒子表面と血液凝固因子との接触、②組織障害などを起点とする血液凝固カスケードの活性化と急性毒性の発現との連関解析を進めており、これらの知見を有効活用することで、生体にとって有害な作用を及ぼさない安全かつ有効な非晶質ナノシリカの創製が実現するものと考えている。

2. NanoTox 解析の新技術

これまでに我々は、透過型電子顕微鏡を用いて

nSPの体内動態追跡を進めている。電子顕微鏡解析においては、形状やサイズを指標にnSPの局在を解析することが可能であるが、生体中に存在する微粒子（カルシウムなど）や染色によって生ずる不純物としての微粒子との区別が付きにくいなどの課題を抱えている。そこで筆者らは、X線元素分析装置付き電子顕微鏡（EDX-TEM）を用いて、生体中nSPの同定を試みた。本装置を用いることで、nSP由来のケイ素原子シグナルを検出することができ、視覚的な局在観察と同時にnSPの同定が可能である。nSP70を静脈内投与したマウスの肝臓を用いてEDX-TEM解析を実施した結果（図8）、肝臓組織切片中に認められた黒いドットからケイ素由来のシグナルを検出することが出来た。この結果から、静脈内投与したnSP70が確かに肝臓まで到達することが裏付けられた。以上、X線分析装置を併用することでnSP、ひいてはNMの生体内細胞内局在を極めて詳細に追跡できる基盤技術の確立に成功した。

一方で、EDX-TEMを解析では、NMの生体内動態を定量的に解析する事は不可能である。NMの曝露量や体内吸収量、閾値、無作用量などの算出に当たっては、NMの定量解析法の開発が必要不可欠である。そこで本年度は、NMの定量解析法の確立を目指して、ICP-AESの有用性を検証した。ICP-AESはICPによってサンプルを原子化・熱励起し、これが基底状態に戻る際の発光スペクトルから元素の同定・定量を行う方法である。特定の原子から発するスペクトルから目的原子の定量解析が可能であるため、NMの定量解析にも応用できるものと考えられた。本年度は、nSP70を静脈内投与したマウスの肝臓を用いて予備検討を実施した。その結果、2 mgのnSP70を投与したマウスの肝臓には、およそ0.8 mgのケイ素が存在していることが明らかとなった。これは1.7 mgのnSP70に相当し、投与したnSP70の内85%が肝臓に到達することが明らかとなった。また、本手法を用いた際の組織重量1 gあたりのケイ素の検出限界は0.5 mgであり、検出感度の改善が必要であるものと考えられた。現在、本手法を適用して曝露量や体内吸収量の解析を進めると共に、検出感度の改善を進めている。

これらX線元素分析法の感度を向上させる方策として、X線強度を増強させることが有力である。これによって、必然的に目的元素由来のシグナルが強くなるため、検出感度が向上するものと予想される。そこで、本年度は世界最高峰の放射光施設であるSPring-8の光源を利用した走査型X線顕微鏡解析を実施し、検出感度の増強が可能か否かを検証した。本検討では、酸化チタンを静脈内投与したマウスの肺および肝臓を使用した。これらのマウスの肺では酸化チタンの存在を位相差顕微鏡にて視覚的に確認出来る一方で、肝臓では確認することは出来ない。まず、酸化チタンを視認できる肺の走査型X線顕微鏡解析を実施したところ、位相差顕微鏡で黒い影として見えた物質が、確かに酸化チタンであることが確認出来た。また、酸化チタンの存在を視認できない肝臓を解析したところ、予想に反して所々に酸化チタン由来のシグナルが検出された。これらの結果は、走査型X線顕微鏡を適用することで酸化チタンの生体内局在をより高感度に解析できることを示唆している。現在、SPring-8の放射光を利用した、NMの定量解析法の確立を進めている。

E. 結論

以上、本研究ではNanoTox解析におけるNM-生体高分子間相互作用の重要性を示し、これらの観点がnSP70誘発性急性致死毒性発現機構を解析するにあたって極めて有用なアプローチになり得ることを示した。現在、nSPのみならず、SPの表面修飾体、さらには酸化チタンやフラーレン、カーボンナノチューブをはじめとする種々のNMの免疫応答・細胞分化・生体/細胞内輸送といった生体応答に与える影響やNMの生体/細胞内動態をNM-生体高分子間相互作用の視点から解析すると共に、NMと常在菌（皮膚常在菌や腸内細菌）との相互作用の観点からもNanoTox解析を

進めている。また各種疾病の悪化とNMの関係や、老化とNM、医薬品や化粧品、食品成分とNMとの相互作用などについても検討を開始している。特に機能性化粧品の機能に及ぼすNMの影響は、抗原性などの観点からも非常に重要であろう。今回のnSPの解析例に見られるように、NM特有の物性-動態-安全性連関を、NMと相互作用する特定の分子から解析・説明することはNanoToxに対してより明確で分かり易い科学的根拠を与えるものと考えられる。従って、NM-生体高分子間相互作用を指標にしたNanoTox解析研究は、一般消費者-行政担当者-安全性評価研究者間のリスクコミュニケーションやNMの使用規制や設計指針の策定を容易化し、これらを基盤とすることで将来的にはNMの安全性確保に適うリスクマネジメントの実現に繋がるものと期待している。

F. 健康危険情報

該当なし

G. 研究発表

①論文発表

1. Yoshikawa T., Nabeshi H., Matsuyama K., Nakazato Y., Imazawa T., Yoshioka Y., Abe Y., Kawai Y., Mayumi T., Itoh N., Tsunoda S., Tsutsumi Y. : In vitro nanotoxicological study of silica nanoparticles using dermal cell lines., Toxicol. Lett., 189S: S179-180, 2009. (Supplement)
2. Nabeshi H., Yoshikawa T., Matsuyama K., Nakazato Y., Imazawa T., Yoshioka Y., Abe Y., Kawai Y., Mayumi T., Itoh N., Tsunoda S., Tsutsumi Y. : Evaluation of size-dependent acute toxicity and toxicokinetics of amorphous nanosilicas., Toxicol. Lett., 189S: S181-182, 2009. (Supplement)
3. Matsuyama K., Yoshikawa T., Nabeshi H., Nakazato Y., Imazawa T., Yoshioka Y., Abe Y., Kawai Y., Mayumi T., Itoh N., Tsunoda S., Tsutsumi Y. : Evaluation of size-dependent intracellular distribution and genotoxicity of amorphous nanosilicas in human keratinocytes., Toxicol. Lett., 189S: S183, 2009. (Supplement)
4. Nakazato Y., Yoshikawa T, Nabeshi H, Matsuyama K, Imazawa T, Yoshioka Y, Abe Y., Kawai Y., Mayumi T., Itoh N., Tsunoda S., Tsutsumi Y. : Differential acute toxicity and toxicokinetics of amorphous nanosilicas: The role of surface physicochemical properties., Toxicol. Lett., 189S: S183, 2009. (Supplement)
5. Nabeshi H., Yoshikawa T., Matsuyama K., Nakazato Y., Arimori A., Isobe M., Tochigi S., Kondoh S., Hirai T., Akase T., Yamashita T., Yamashita K., Yoshida T., Nagano K., Abe Y., Yoshioka Y., Kamada H., Imazawa T., Itoh N., Tsunoda S., Tsutsumi Y. : Size-dependent cytotoxic effects of amorphous silica nanoparticles on Langerhans cells., Pharmazie., in press.

【総説・その他】

1. Yoshikawa T, Nabeshi H, Yoshioka Y. : Evaluation of Biological Influence of Nano-materials using Toxicokinetic and Toxicoproteomic Approach., Yakugaku Zasshi, 128(12):1715-1725, 2008.
2. 吉岡靖雄、吉川友章、角田慎一：医薬品・香粧品におけるナノマテリアルの安全性評価、Pharma-VISION 第12号: 14-18, 2008.
3. Nabeshi H., Yoshikawa T., Imazawa T., Tsunoda S., Tsutsumi Y. : Evaluation of Biological Influence of Nano-materials

using Toxicokinetics and Toxicoproteomcs., Yakugaku Zasshi, in press.

4. 吉川友章, 吉岡靖雄, 堤　康央：ナノ粒子の細胞内取り込みと経皮浸透.,ナノ材料のリスク評価と安全性対策, フロンティア出版, in press.
5. 化粧品ナノマテリアルの安全性評価の現状と技術的課題～安全なナノマテリアルの開発支援に向けて～, コスメティックステージ総説, in press.

② **学会発表**

【シンポジウム等：合計 1 件】

1. 吉川友章：ナノマテリアルの相互作用解析と急性毒性・肝毒性機構の追求., 日本薬学会第 130 年会, 岡山, 2010 年 3 月.

【国内学会発表：合計 21 件】

1. 鍋師裕美, 吉川友章, 仲里泰太郎, 松山恵吾, 吉岡靖雄, 阿部康弘, 眞弓忠範, 伊藤徳夫, 角田慎一, 堤　康央：食品添加物としてのナノシリカの物性と生体影響の連関解析., 日本食品衛生学会第 97 回学術講演会, 中央区（東京）, 2009 年 5 月.
2. 松山恵吾, 吉川友章, 鍋師裕美, 仲里泰太郎, 吉岡靖雄, 阿部康弘, 河合裕一, 眞弓忠範, 伊藤徳夫, 角田慎一, 堤　康央：香粧品素材としての非結晶性ナノシリカの皮膚細胞に対する遺伝毒性評価と安全確保に向けて., 第 34 回日本香粧品学会, 港区（東京）, 2009 年 6 月.
3. 山下浩平, 吉岡靖雄, 東阪和馬, 萱室裕之, 吉田徳幸, 阿部康弘, 吉川友章, 河合裕一, 眞弓忠範, 伊藤徳夫, 角田慎一, 堤　康央:非結晶性ナノシリカの生殖発生毒性に関する検討., 日本先天異常学会第 49 回学術集会, 鹿児島（鹿児島）, 2009 年 6 月.
4. 鍋師裕美, 吉川友章, 仲里泰太郎, 松山恵吾, 今澤孝喜, 吉岡靖雄, 阿部康弘, 河合裕一, 眞弓忠範, 伊藤徳夫, 角田慎一, 堤　康央:ナノシリカの in vitro 安全性評価における血清蛋白質の作用の解析., 第 36 回日本トキシコロジー学会学術年会, 盛岡（岩手）, 2009 年 7 月.
5. 松山恵吾, 吉川友章, 鍋師裕美, 仲里泰太郎, 吉岡靖雄, 今澤孝喜, 阿部康弘, 河合裕一, 眞弓忠範, 伊藤徳夫, 角田慎一, 堤　康央:非結晶性ナノシリカの皮膚細胞に対する遺伝毒性作用., 第 36 回日本トキシコロジー学会学術年会, 盛岡（岩手）, 2009 年 7 月.
6. 仲里泰太郎, 吉川友章, 鍋師裕美, 松山恵吾, 吉岡靖雄, 今澤孝喜, 阿部康弘, 河合裕一, 眞弓忠範, 伊藤徳夫, 角田慎一, 堤　康央:非結晶性ナノシリカの表面物性と有害性発現との連関評価., 第 36 回日本トキシコロジー学会学術年会, 盛岡（岩手）, 2009 年 7 月.
7. 山下浩平, 吉岡靖雄, 東阪和馬, 萱室裕之, 吉田徳幸, 阿部康弘, 吉川友章, 河合裕一, 眞弓忠範, 伊藤徳夫, 角田慎一, 堤　康央:非結晶性ナノシリカの生殖発生毒性に関する検討., 第 36 回日本トキシコロジー学会学術年会, 盛岡（岩手）, 2009 年 7 月.
8. 吉田徳幸, 吉岡靖雄, 中西亮介, 山下浩平, 東阪和馬, 萱室裕之, 阿部康弘, 吉川友章, 河合裕一, 眞弓忠範, 伊藤徳夫, 角田慎一, 堤　康央: 都市大気粉塵のアレルギー疾患誘発メカニズムの解析., 第 36 回日本トキシコロジー学会学術年会, 盛岡（岩手）, 2009 年 7 月.
9. 東阪和馬, 吉岡靖雄, 山下浩平, 萱室裕之, 吉田徳幸, 阿部康弘, 吉川友章, 河合裕一, 眞弓忠範, 伊藤徳夫, 角田慎一, 堤　康央:カーボンナノチューブの発癌リスクに関する基礎的検討., 第 36 回日本トキシコロジー学会学術年会, 盛岡（岩手）, 2009 年 7 月.
10. 今澤孝喜, 有森亮裕, 磯部将彰, 鍋師裕美, 物部容子, 角田慎一, 堤　康央: ナノマテリ

アルのマウスにおける細胞内局在性に関する研究., 第 36 回日本トキシコロジー学会学術年会, 盛岡（岩手）, 2009 年 7 月.

11. 角田慎一, 吉川友章, 鍋師裕美, 赤瀬貴憲, 長野一也, 阿部康弘, 吉岡靖雄, 今澤孝喜, 向　洋平, 岡田直貴, 中川晋作, 堤　康央:ナノマテリアルの安全確保に向けて-1　ナノマテリアルの経皮吸収性・体内動態と急性毒性・肝毒性., 第 98 回日本食品衛生学会学術講演会, 函館（北海道）, 2009 年 10 月.
12. 吉川友章, 鍋師裕美, 平井敏郎, 長野一也, 阿部康弘, 吉岡靖雄, 角田慎一, 近藤昌夫, 八木清仁, 堤　康央：ナノマテリアルの安全確保に向けて-2　ナノマテリアルの相互作用解析と急性毒性・肝毒性機構の追求., 第 98 回日本食品衛生学会学術講演会, 函館（北海道）, 2009 年 10 月.
13. 阿部康弘, 吉川友章, 鍋師裕美, 松山恵吾, 近藤小百合, 長野一也, 吉岡靖雄, 今澤孝喜, 角田慎一,堤　康央:ナノマテリアルの安全確保に向けて-3　ナノマテリアルの細胞内動態と遺伝毒性., 第 98 回日本食品衛生学会学術講演会, 函館（北海道）, 2009 年 10 月.
14. 吉岡靖雄, 森重智弘, 吉田徳幸, 稲倉　裕, 藤村真穂, 鍋師裕美, 阿部康弘, 長野一也, 吉川友章, 今澤孝喜, 角田慎一, 向　洋平, 岡田直貴, 堤　康央, 中川晋作：ナノマテリアルの安全確保に向けて-4　ナノマテリアルの動態特性と免疫毒性., 第 98 回日本食品衛生学会学術講演会, 函館（北海道）, 2009 年 10 月.
15. 長野一也, 吉岡靖雄, 山下浩平, 東阪和馬, 森下裕貴, 鍋師裕美, 阿部康弘, 吉川友章, 角田慎一, 斉藤　滋, 河合裕一, 眞弓忠範, 堤　康央：ナノマテリアルの安全確保に向けて-5　ナノマテリアルの動態特性と生殖発生毒性., 第98回日本食品衛生学会学術講演会, 函館（北海道）, 2009 年 10 月.
16. 鍋師裕美, 吉川友章, 仲里泰太郎, 栃木彩恵子, 長野一也, 阿部康弘, 吉岡靖雄, 今澤孝喜, 角田慎一, 堤　康央：ナノマテリアルの安全確保に向けて-6　表面修飾ナノシリカの安全性評価と安全なナノマテリアルの開発支援., 第 98 回日本食品衛生学会学術講演会, 函館（北海道）, 2009 年 10 月.
17. 吉川友章, 鍋師裕美, 仲里泰太郎, 松山恵吾, 栃木彩恵子, 近藤小百合, 平井敏郎, 伊藤徳夫, 堤　康央：薬学領域におけるナノマテリアルの安全性確保研究　～ナノマテリアルの体内相互作用解析と安全性との連関追求～., 第 59 回日本薬学会近畿支部総会・大会, 東大阪（大阪）, 2009 年 10 月.
18. Yoshida T., Yoshioka Y., Kayamuro H., Yamashita K., Higashisaka K., Fujimura M., Abe Y., Kawai Y., Mayumi T., Yoshikawa T., Itoh N., Tsunoda S., Tsutsumi Y. : Urban aerosol directly stimulates antigen presentation cells in vitro and cause airway inflammation in vivo., 第 39 回 日本免疫学会学術集会, 大阪（大阪）, 2009 年 12 月.
19. 山下浩平, 吉岡靖雄, 東阪和馬, 森下裕貴, 吉田徳幸, 藤村真穂, 鍋師裕美, 長野一也, 阿部康弘, 吉川友章, 鎌田春彦, 伊藤徳夫, 眞弓忠範, 角田慎一, 堤　康央 : 安全確保に向けた非晶質ナノシリカの生殖発生毒性に関する検討., ファーマバイオフォーラム 2009, 名古屋（愛知）, 2009 年 11 月.
20. 平井敏郎, 吉川友章, 鍋師裕美, 仲里泰太郎, 松山恵吾, 栃木彩恵子, 近藤小百合, 赤瀬貴憲, 長野一也, 阿部康弘, 吉岡靖雄, 鎌田春彦, 伊藤徳夫, 角田慎一, 堤　康央: 樹状細胞を標的としたナノシリカの経皮安全性評価., 日本薬学会　第 130 年会, 岡山（岡山）, 2010 年 3 月.
21. 栃木彩恵子, 吉川友章, 鍋師裕美, 仲里泰太郎, 松山恵吾, 平井敏郎, 近藤小百合, 赤瀬貴憲, 長野一也, 阿部康弘, 吉岡靖雄, 鎌田

春彦, 伊藤徳夫, 角田慎一, 堤　康央： 血液凝固系に着目した非晶質ナノシリカの安全性評価., 日本薬学会　第 130 年会, 岡山（岡山）, 2010 年 3 月.

【国際学会発表：合計 4 件】

1. Yoshikawa T., Nabeshi H., Matsuyama K., Nakazato Y., Imazawa T., Yoshioka Y., Abe Y., Kawai Y., Mayumi T., Itoh N., Tsunoda S., Tsutsumi Y. : In vitro nanotoxicological study of silica nanoparticles using dermal cell lines., The 46th Congress of the European Societies of Toxicology (EuroTox), Dresden (Germany), 13-16 September, 2009.
2. Nabeshi H., Yoshikawa T., Matsuyama K., Nakazato Y., Imazawa T., Yoshioka Y., Abe Y., Kawai Y., Mayumi T., Itoh N., Tsunoda S., Tsutsumi Y. : Evaluation of size-dependent acute toxicity and toxicokinetics of amorphous nanosilicas., The 46th Congress of the European Societies of Toxicology (EuroTox), Dresden (Germany), 13-16 September, 2009.
3. Matsuyama K., Yoshikawa T., Nabeshi H., Nakazato Y., Imazawa T., Yoshioka Y., Abe Y., Kawai Y., Mayumi T., Itoh N., Tsunoda S., Tsutsumi Y. : Evaluation of size-dependent intracellular distribution and genotoxicity of amorphous nanosilicas in human keratinocytes., The 46th Congress of the European Societies of Toxicology (EuroTox), Dresden (Germany), 13-16 September, 2009.
4. Nakazato Y., Yoshikawa T, Nabeshi H, Matsuyama K, Imazawa T, Yoshioka Y, Abe Y., Kawai Y., Mayumi T., Itoh N., Tsunoda S., Tsutsumi Y. : Differential acute toxicity and toxicokinetics of amorphous nanosilicas: The role of surface physicochemical properties., The 46th Congress of the European Societies of Toxicology (EuroTox), Dresden (Germany), 13-16 September, 2009.

H. 知的財産権の出願・登録状況

① 特許取得

該当なし

② 実用新案登録

該当なし

その他

研究協力者

該当なし

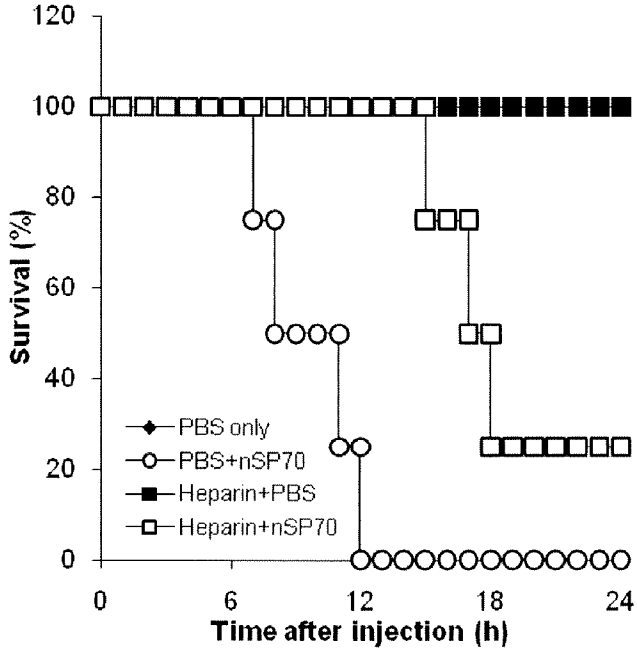

図 1. 抗凝固剤ヘパリン前投与マウスを用いた生存率の評価
BALB/c マウスに 100 IU/匹のヘパリン溶液あるいは PBS を尾静脈内より投与し、2 分後に nSP70（2 mg/匹）を尾静脈内投与した際の生存率を経時的に 24 時間まで評価した。

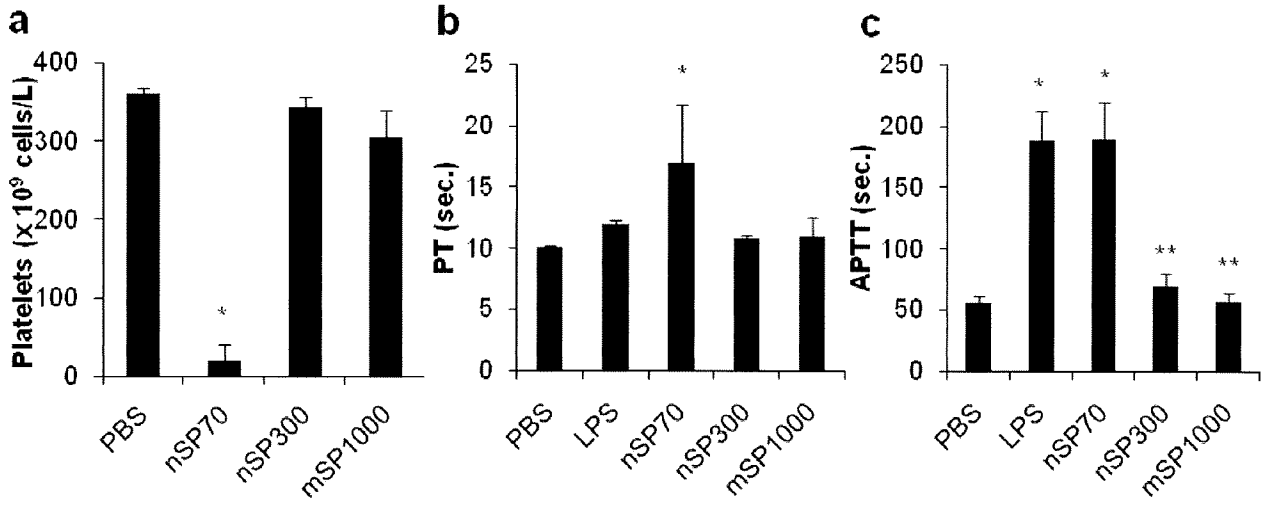

図 2. ナノシリカ投与マウスの血液凝固検査
各粒子サイズのシリカを 2 mg/匹で尾静脈内投与し、5 時間後に血液を回収した。全血の一部を用いて血小板数を計測した(a)。残りの血液を 1750 × g、15 分間、遠心分離して得られた血漿を用いてプロトロンビン時間（PT, b）および活性化部分トロンボプラスチン時間（APTT, c）を測定した。*P<0.01 vs PBS, **P<0.01 vs nSP70 (Bonferroni)

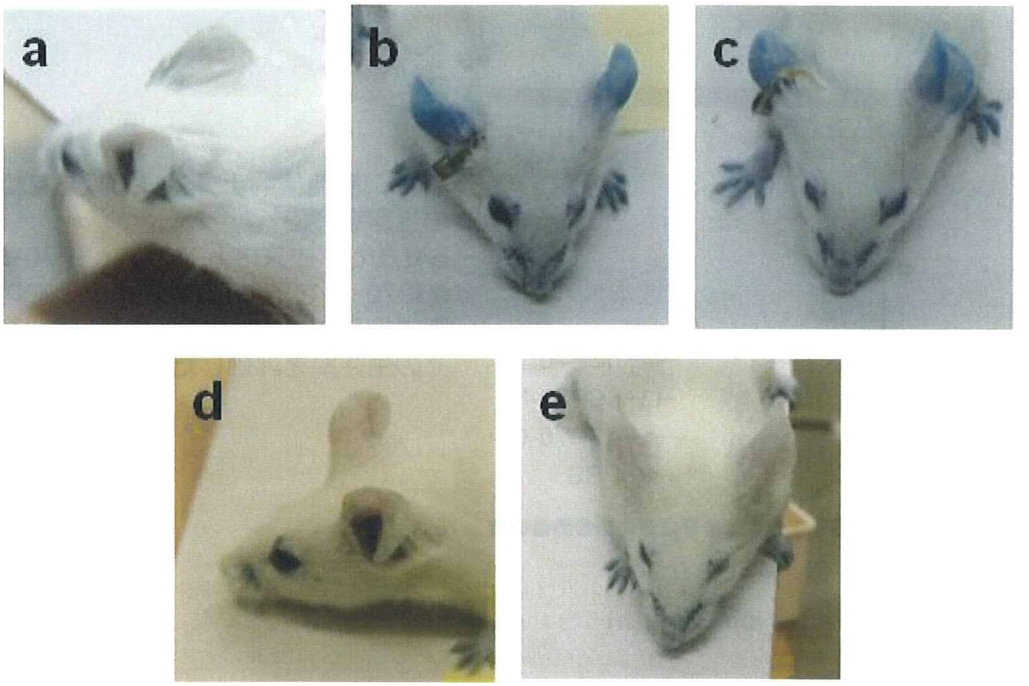

図 3. ナノシリカ投与マウスの血管透過性試験
BALB/c マウスに PBS（a）あるいは、nSP70（b）、nSP100（c）、nSP300（d）、mSP1000（e）をそれぞれ 2 mg/ 匹ずつ尾静脈内投与した。シリカ投与 1 時間後に、2%エバンスブルー溶液を 80 μl/匹ずつ尾静脈内より投与し、30 分後に耳介の色調変化を撮影した。

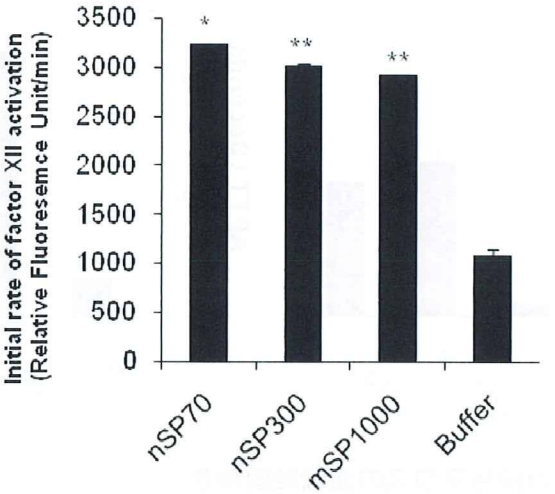

図 4. ナノシリカの血液凝固第 XII 因子活性化能の評価
37°C に加温しておいた 1 ml の Buffer（pH8.0 Tris-HCl in 0.15 M NaCl, 1 mMCaCl2, 0.1 mg/ml bovine serum albumin）に、血液第XIIa 因子基質（PEPTIDE INSTITUTE, Inc, Japan）を 20 μl 加えた。これとは別に 100 μl のヒト血漿に各 nSP（25 mg/ml, 0.4 mg/ml）を 100 μl を加え攪拌した。この血漿と nSP との混合液を、先程の Buffer と基質の混合液に加え、測定波長 380 nm、参照波長 440 nm で測定した。横軸に反応後の経過時間、縦軸に蛍光強度を取り、各サンプルのグラフが直線状態になる時間範囲について近似曲線を引くことで、その傾きから酵素活性化の初速度 V0 を算出した。*P<0.01 vs PBS, **P< 0.01 vs nSP70

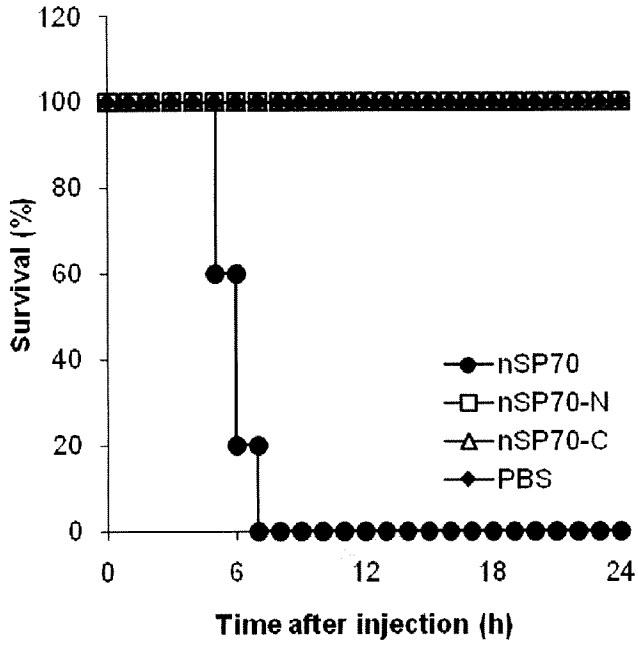

図 5. 表面修飾ナノシリカ投与マウスの生存率評価
BALB/c マウスに nSP70、nSP70-N、nSP70-C をそれぞれ 2 mg/匹で尾静脈内投与し、1 時間ごとに 24 時間後までの生存率を評価した。

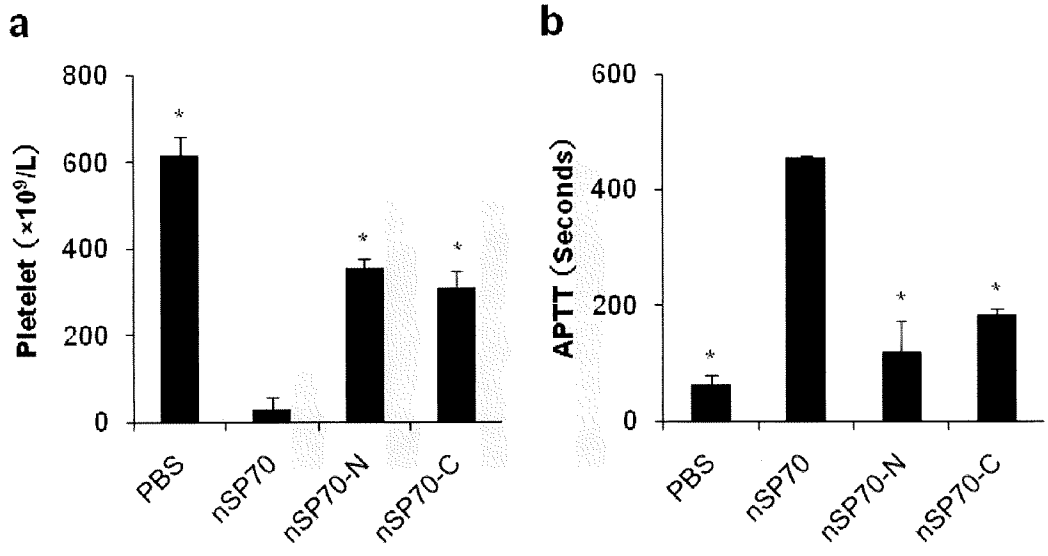

図 6. 表面修飾ナノシリカ投与マウスの血液凝固検査
各粒子サイズのシリカを 2 mg/匹で尾静脈内投与し、5 時間後に血液を回収した。全血の一部を用いて血小板数を計測した(a)。残りの血液を 1750 × g、15 分間、遠心分離して得られた血漿を用いて活性化部分トロンボプラスチン時間 (APTT, b) を測定した。*P<0.01 vs PBS, *P<0.01 vs nSP70 (Fisher's PLSD)

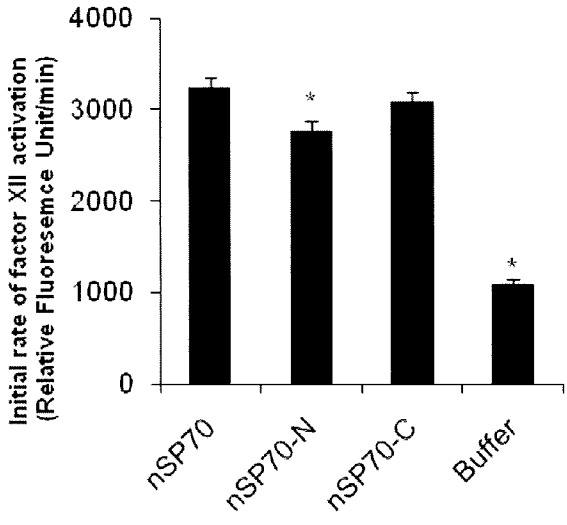

図 7. 表面修飾ナノシリカの血液凝固第 XII 因子活性化能の評価

37°C に加温しておいた 1 ml の Buffer（pH8.0 Tris-HCl in 0.15 M NaCl, 1 mMCaCl2, 0.1 mg/ml bovine serum albumin）に、血液第XIIa因子基質（PEPTIDE INSTITUTE, Inc, Japan）を 20 µl 加えた。これとは別に 100 µl のヒト血漿に各 nSP（25 mg/ml, 0.4 mg/ml）を 100 µl を加え攪拌した。この血漿と nSP との混合液を、先程の Buffer と基質の混合液に加え、測定波長 380 nm、参照波長 440 nm で測定した。横軸に反応後の経過時間、縦軸に蛍光強度を取り、各サンプルのグラフが直線状態になる時間範囲について近似曲線を引くことで、その傾きから酵素活性化の初速度 V0 を算出した。*P<0.001 vs nSP70 (Fisher's PLSD)

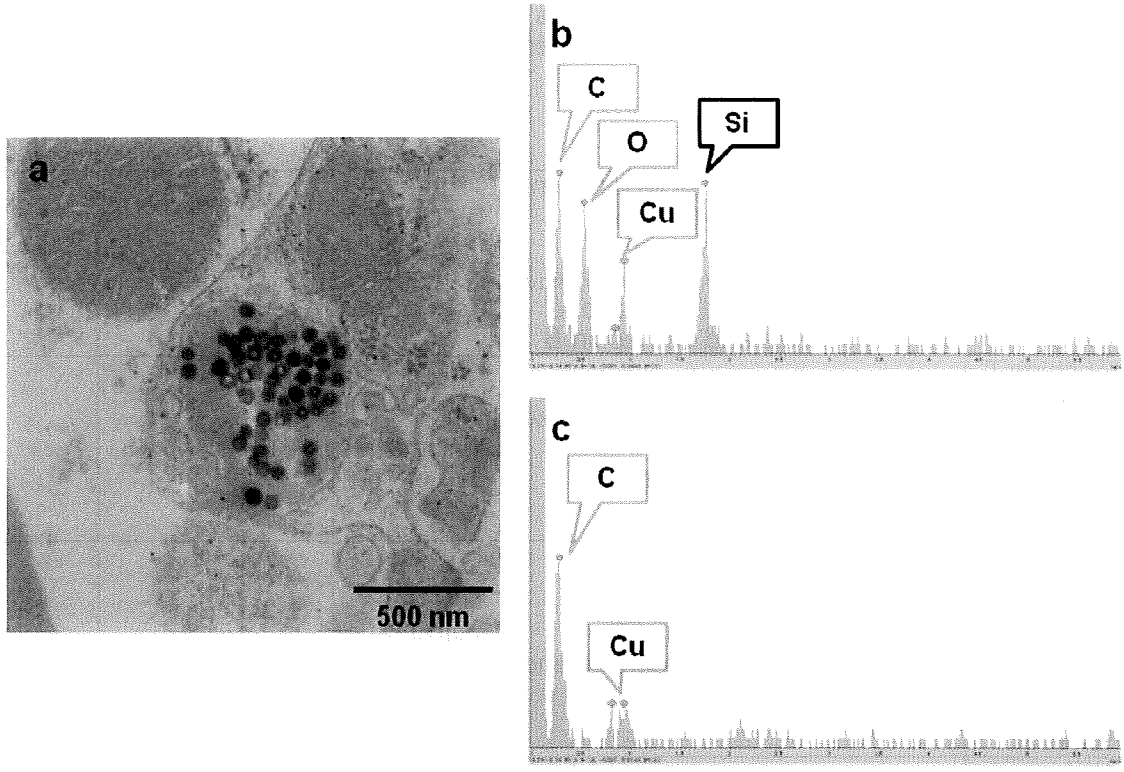

図 8. エネルギー分散型 X 線検出器付き透過型電子顕微鏡（EDX-TEM）を用いたナノシリカの体内局在解析

BALB/c マウスに nSP70 を 2 mg/匹で尾静脈内投与し、5 時間後に肝臓を摘出し、2.5%グルタルアルデヒドで固定した。その後、洗浄・脱水・樹脂置換し、Epon-812 で包埋した。この組織ブロックを薄切し、得られた超薄切片をエネルギー分散型 X 線装置（EDX）を装着した透過型電子顕微鏡（TEM; 日立ハイテクノロジーズ）を用いて解析した。a, 肝クッパー細胞の透過型電子顕微鏡像、b, 粒子部分（a の黒点部分）の EDX 分析スペクトル、c, バックグラウンドの EDX 分析スペクトル。C：炭素、O：酸素、Cu：銅、Si：ケイ素。

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	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
吉川友章, 吉岡靖雄, 堤　康央	非晶質ナノシリカの経皮吸収性/生体内動態と安全性との連関追求	亘理文夫（北海道大学大学院歯学研究科）	ナノ材料のリスク評価と安全性対策	フロンティア出版	日本	In press	

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Nabeshi H., Yoshikawa T., Abe Y., Yoshioka Y., Imazawa T., Tsunoda S., Tsutsumi Y. et al.	Size-dependent cytotoxic effects of amorphous silica nanoparticles on Langerhans cells.	Pharmazie.		In press	
Morishige T., Yoshioka Y., Tsunoda S., Tsutsumi Y., Nakagawa S. et al.	Titanium dioxide induces different levels of IL-1beta production dependent on its particle characteristics through caspase-1 activation mediated by reactive oxygen species and cathepsin B	Biochem. Biophys. Res. Commun.		In press	
Morishige T., Yoshioka Y., Tsunoda S., Tsutsumi Y., Nakagawa S. et al.	Cytotoxicity of amorphous silica particles against macrophage-like THP-1 cells depends on particle-size and surface properties.	Pharmazie.		In press	
Yamashita K., Yoshioka Y., Abe Y., Yoshikawa T., Tsunoda S., Tsutsumi Y. et al.	Carbon nanotubes elicit DNA damage and inflammatory response relative to their size and shape.	Inflammation		In press	
Nishimori H., Kondoh M., Yagi K. et al.	Histological analysis of 70-nm silica particles-induced chronic toxicity in mice.	Eur. J. Pharm. Bipharm.	72	626-629	2009
Nishimori H., Kondoh M., Yagi K. et al.	Silica nanoparticles as hepatotoxicants.	Eur. J. Pharm. Bipharm.	72	496-501	2009

Nishimori H., Kondoh M., Yagi K. et al.	Influence of 70-nm silica particles in mice with cisplatin or paraquat-induced toxicity.	Pharmazie	64	395-397	2009

Graduate School of Pharmaceutical Sciences[1], Osaka University; Laboratory of Pharmaceutical Proteomics[2], National Institute of Biomedical Innovation; The Center for Advanced Medical Engineering and Informatics[3], Osaka University; Bioresources Research[4], Laboratory of Common Apparatus, National Institute of Biomedical Innovation, Osaka, Japan

Size-dependent cytotoxic effects of amorphous silica nanoparticles on Langerhans cells

H. NABESHI[1,2,*], T. YOSHIKAWA[1,2,*], K. MATSUYAMA[1,2], Y. NAKAZATO[1,2], A. ARIMORI[1,2], M. ISOBE[1,2], S. TOCHIGI[1,2], S. KONDOH[1,2], T. HIRAI[1,2], T. AKASE[1,2], T. YAMASHITA[1,2], K. YAMASHITA[1,2], T. YOSHIDA[1,2], K. NAGANO[2], Y. ABE[2], Y. YOSHIOKA[2,3], H. KAMADA[2,3], T. IMAZAWA[4], N. ITOH[1], S. TSUNODA[1,2,3,**], Y. TSUTSUMI[1,2,3]

Received August 10, 2009, accepted August 14, 2009

Tomoaki Yoshikawa, Ph.D. and Yasuo Tsutsumi, Ph.D., Department of Toxicology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565-0871, Japan
tomoaki@phs.osaka-u.ac.jp
**These authors contributed equally to the work.*
***Shin-ichi Tsunoda, Ph.D., Laboratory of Pharmaceutical Proteomics, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki, Osaka 567-0085, Japan.*

Pharmazie 65: 199–201 (2010) *doi: 10.1691/ph.2010.9268*

Amorphous silica nanoparticles (nSPs), are widely used in medicines, cosmetics and food. However, due to their reduced particle size they are suspected to pose new risks induced by changes in biological reactivity and kinetics, which differ from those of bulk materials. In a previous study, we showed that silica particles with a diameter of 70 nm penetrated the stratum corneum (SC) of mouse skin and were taken up by living cells such as keratinocytes and Langerhans cells. To clarify the relationship between particle size, distribution and cellular response, we have evaluated size-dependent intracellular localization and cytotoxicity of silica particles, using the mouse epidermal Langerhans cell line XS52. On treatment with silica particles of diameters 70, 300, and 1000 nm, cellular uptake and cytotoxicity increased with reduction in particle size. These results suggest that smaller sized silica particles induced greater cytotoxicity against Langerhans cells, which was correlated with the quantity of particle uptake into the cells.

1. Introduction

The recent development of nanoscale engineering represents a current dynamic area of interdisciplinary research, incorporating nanomaterials (NMs) into a diverse product matrix such as diagnostics, food additives and cosmetics. Because amorphous silica nanoparticles (nSPs) and titanium oxide nanoparticles, etc. are colorless and reflect ultraviolet more efficiently than micro-sized particles, nSPs and titanium oxide nanoparticles are already used as cosmetic vehicles or functional ingredients in many cosmetics such as foundation creams and sunscreens. However, because NMs may possess novel properties, kinetics, and biological effects different from those of micro size bulk materials, their potential harmful effects on humans are raising concerns about their safety. Thus, there is an urgent need for risk assessment of NMs. To achieve this, it is most important to analyze the relationship between particle-size parameters, cellular distribution and biological effects, allowing prediction and avoidance of risk in using NMs.

In a previous study, we showed that silica particles with a diameter of 70 nm penetrated the stratum corneum (SC) of mouse skin and were taken up by living cells such as keratinocytes and Langerhans cells. So, to reveal the relationship between particle size, distribution, and cellular response, we evaluated size-dependent intracellular localization and cytotoxicity of silica particles, using the mouse epidermal Langerhans cell line XS52.

2. Investigations, results and discussion

To assess cellular uptake of nSPs, we observed XS52 cells treated with 100 μg/ml nSP70, nSP300 and mSP1000 using transmission electron microscopy (TEM). We found that nSP300 and mSP1000 were located in cytoplasm only (Fig. 1c and d), while nSP70 was surprisingly located in nucleus as well as cytoplasm (Fig. 1a and b). Furthermore the quantity of silica particles taken up by the cells increased as particle size decreased. These results suggested that the uptake and localization of silica particles altered with particle size.

We next investigated biological effects of various-sized silica particles in XS52 cells. To assess the effect of treatments with nSPs on cellular proliferation, the [^{3}H]-thymidine incorporation assay was performed. As shown in Fig. 2, XS52 cell proliferation was dose-dependently inhibited by treatment with silica particles of all sizes. IC50 values for nSP70, nSP300 and mSP1000 were 4.2, 32.6, and 75.0 μg/ml, respectively. These results showed that the growth of XS52 cells was more strongly inhibited by smaller-sized nSP.

To study the mechanism responsible for the effects on XS52 cells treated with various-sized silica particles, we measured the quantity of lactate dehydrogenase (LDH) released. LDH is released into culture medium after the cellular membrane disruption that constitutes the last step of the in vitro cell death process. After 24 h of exposure (Fig. 3), no LDH release was observed in mSP1000-treated cells, while dose-dependent LDH

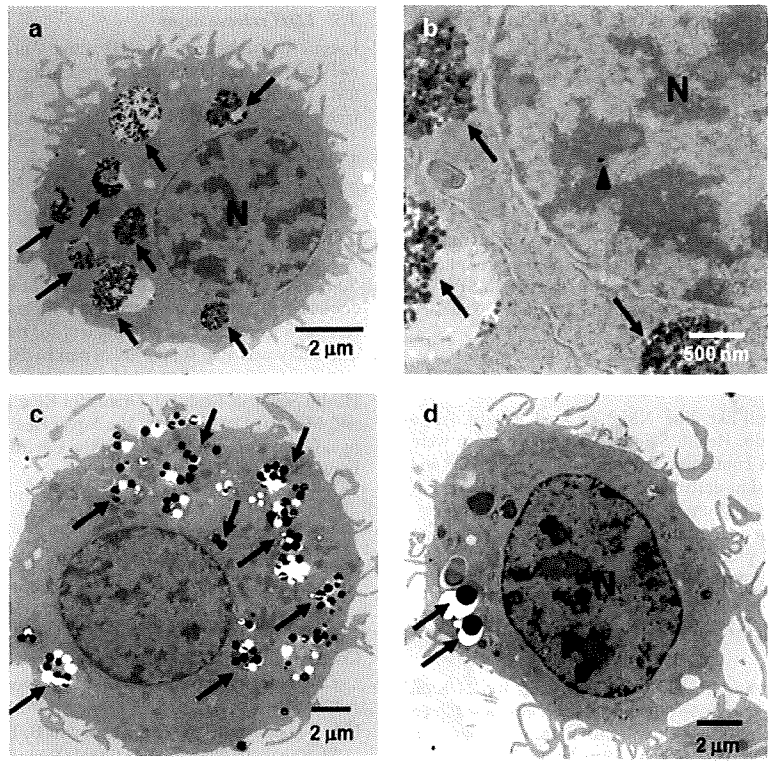

Fig. 1: Localization of silica particles in XS52 cells (arrows). Cells were treated for 24 h with nSP70 (a and b), nSP300 (c) and mSP1000 (d). nSP300 and mSP1000 were located in cytoplasm only. On the other hand, nSP70 was located in the nucleus as well as in cytoplasm (b, arrow head). Scale bars; 2 µm (a, c and d), 500 nm (b)

release was observed in nSP70- and nSP300-treated cells. The highest LDH release was recorded on treatment with 30 µg/ml nSP70 (193 ± 6.8% of control). This result therefore indicated that the cytotoxicity to XS52 cells may be due to cellular membrane damage. Consequently, it appears that the difference in the quantity of silica particles taken up by the cells may explain the size-dependent toxicity to XS52 cells.

As reported elsewhere, we had shown that nSP70 penetrated the stratum corneum (SC) of mouse skin and was taken up by living Langerhans cells (Nabeshi et al. 2010). Furthermore, in the present study we showed that the difference in the quantity of silica particles taken up by the cells was linked to size-dependent toxicity and nSP70 taken up by Langerhans cells entered the nucleus. Thus, our previous and present results suggest that transdermal exposure to nSPs may (i) risk dysfunction of Langerhans cells, as shown by the cytotoxicity to XS52 cells, (ii) induce immune disruption by altering the immune response (Tinkle et al. 2003; Fifis et al. 2004) and (iii) induce dysfunction of the nucleus and genotoxicity via aggregation of intranuclear protein or inhibition of RNA transcription (Chen and von Mikecz 2005) following entrance of nSPs into the nucleus.

Collectively, the data obtained in this study offer highly useful information for prediction and avoidance of harmful effects mediated by nSPs used commercially in cosmetics. Thus, cor-

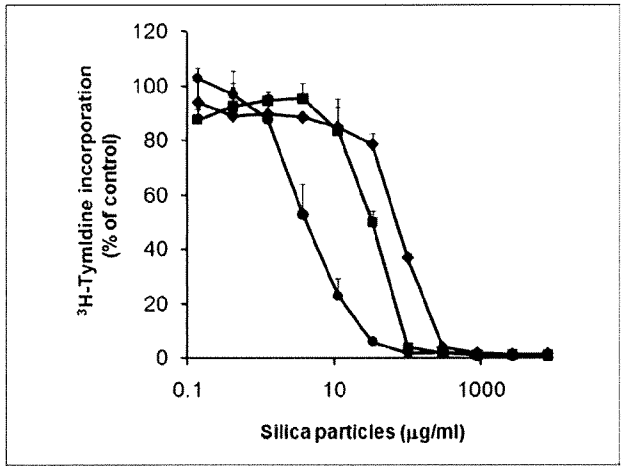

Fig. 2: Effect of various-sized silica particles on proliferation of XS52 cells. The proliferation of cells after incubation with nSP70 (circle), nSP300 (square) and mSP1000 (diamond) for 24 h was evaluated using the [^{3}H]-thymidine incorporation assay. The percentage increase in cell proliferation was calculated relative to the negative control. Data are presented as means ± SD

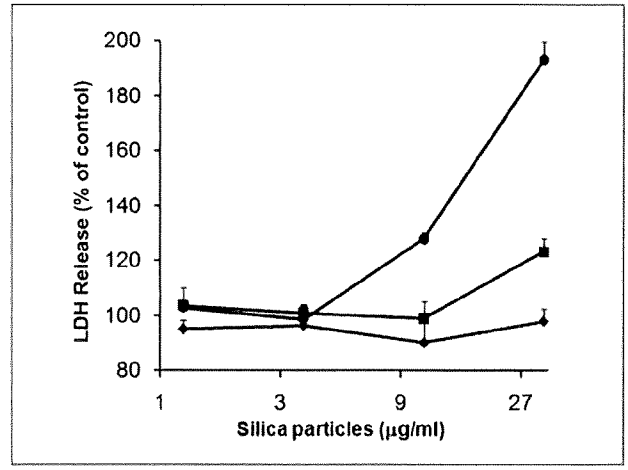

Fig. 3: Effect of silica particles on membrane damage. Cellular membrane damage in XS52 cells after incubation with nSP70 (circle), nSP300 (square) and mSP1000 (diamond) for 24 h was evaluated by the LDH release assay. The percentage cellular membrane damage was calculated relative to the negative (medium) controls. Data are presented as means ± SD

related analysis of physicochemical properties, harmful effects and biodistribution as performed in our study may offer valuable readouts for toxicity of nanomaterials and help to develop non-toxic nanomaterials in the future.

3. Experimental

3.1. Silica particles

Fluorescent (red-F)-labeled silica particle suspensions (25 mg/ml or 50 mg/ml) with a diameter of 70, 300 and 1000 nm (Micromod Partikeltechnologie GmbH, Rostock, Germany; designated nSP70, nSP300, mSP1000, respectively) were used in this study. In each case, silica particles were used after 5 min sonication and 1 min vortexing.

3.2. Cell culture

Cells from the Langerhans cell-like line XS52 (a kind gift of Akira Takashima, University of Toledo, Health Science Campus, Toledo) were expanded in complete medium containing 2 ng/ml murine GM-CSF and 10% culture supernatants from skin-derived stromal NS47 cells (a kind gift of Akira Takashima). Complete medium was RPMI-1640 medium supplemented with 10% heat- inactivated fetal calf serum, 1% non-essential amino acids, 1% L-glutamine, 1 mM sodium pyruvate, 1% 2-mercaptoethanol, 10 mM HEPES buffer, and 1% Antibiotic-Antimycotic Mix stock solution.

3.3. Transmission electron microscopy (TEM) analysis of Langerhans cell line

XS52 cells were cultured with various-sized silica particles for 24 h on chamber slides, then fixed at 4 °C in 2.5% glutaraldehyde and washed three times in 0.1 M phosphate buffer (pH: 7.4); cells were then post-fixed in phosphate-buffered 1% osmium tetroxide for 60 min at 4 °C, dehydrated through a series of ethanol concentrations and embedded in EPON resin (TAAB, Watford, UK). Ultrathin sections were stained with lead citrate and examined under an electron microscope (Hitachi H-7650).

3.4. [^{3}H]-Thymidine incorporation assay

Proliferation of silica particle-treated XS52 cells was measured by [^{3}H]-thymidine incorporation assay. 1×10^4 cells were cultured with varying concentrations of nSPs for 18 h at 37 °C and [^{3}H] thymidine (1 μCi/well) was then added into each well. After a further 6 h, cells were harvested and lysed on glass fiber filter plates using a Cell harvester (PerkinElmer, MA, USA). The filter plates were then dried and counted by standard liquid scintillation counting techniques in a TopCounter (PerkinElmer, MA, USA).

3.5. LDH release assay

Lactate dehydrogenase (LDH) activity of XS52 cells exposed to nSP70, nSP300, mSP1000 was determined using a commercial LDH cytotoxicity test (WAKO, Osaka, Japan) according to the manufacturer's instructions. In brief, 5×10^3 cells were seeded into each well of a 96 well plate. After 24 h incubation, cells were treated with nSP70, nSP300, mSP1000 or 0.2% Tween 20 (positive control). After a further 24 h incubation period, 50 μl of medium overlying cells was used for LDH analysis. Absorption of light at 560 nm was measured using a spectrophotometer.

Acknowledgement: This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and in part by Grants-in-Aid for Scientific Research from the Japanese Society for the Promotion of Science (JSPS). The study was also supported in part by Health Labour Sciences Research Grants from the Ministry of Health, Labor and Welfare of Japan, in part by Health Sciences Research Grants for Research on Publicly Essential Drugs and Medical Devices from the Japan Health Sciences Foundation, in part by a Grant from the Ministry of the Environment, and in part by the Nagai Foundation Tokyo.

References

Bennett CL, van Rijn E, Jung S, Inaba K, Steinman RM, Kapsenberg ML, Clausen BE (2005) Inducible ablation of mouse Langerhans cells diminishes but fails to abrogate contact hypersensitivity. J Cell Biol 169: 569–576.

Chen M, von Mikecz A (2005) Formation of nucleoplasmic protein aggregates impairs nuclear function in response to SiO2 nanoparticles. Exp Cell Res 305: 51–62.

Fifis T, Gamvrellis A, Crimeen-Irwin B, Pietersz GA, Li J, Mottram PL, McKenzie IF, Plebanski M (2004) Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. J Immunol 173: 3148–3154.

Nabeshi H, Yoshikawa T, Matsuyama K, Nakazato Y, Matsuo K, Arimori A, Isobe M, Tochigi S, Kondoh S, Hirai T, Akase T, Yamashita T, Nagano K, Abe Y, Yoshioka Y, Kamada H, Imazawa T, Nakagawa S, Mayumi T, Itoh N, Tsunoda S, Tsutsumi Y (2010) NanoTox studies of nanosilica for ensuring safety-2. In preparation.

Palucka K, Banchereau J (2002) How dendritic cells and microbes interact to elicit or subvert protective immune responses. Curr Opin Immunol 14: 420–431.

Tinkle SS, Antonini JM, Rich BA, Roberts JR, Salmen R, DePree K, Adkins EJ (2003) Skin as a route of exposure and sensitization in chronic beryllium disease. Environ Health Perspect 111: 1202–1208.

European Journal of Pharmaceutics and Biopharmaceutics xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Note

Histological analysis of 70-nm silica particles-induced chronic toxicity in mice

Hikaru Nishimori [a], Masuo Kondoh [a,*], Katsuhiro Isoda [a], Shin-ichi Tsunoda [b,c], Yasuo Tsutsumi [b,c,d], Kiyohito Yagi [a]

[a] Laboratory of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan
[b] Laboratory of Pharmaceutical Proteomics, National Institute of Biomedical Innovation, Osaka, Japan
[c] The Center for Advanced Medicinal Engineering and Informatics, Osaka University, Osaka, Japan
[d] Laboratory of Toxicology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan

ARTICLE INFO

Article history:
Received 10 February 2009
Accepted in revised form 24 March 2009
Available online xxxx

Keywords:
Nanotoxicology
Silica particle
Histological analysis
Liver
Spleen

ABSTRACT

Nano-sized silica is a promising material for disease diagnosis, cosmetics and drugs. For the successful application of nano-sized material in bioscience, evaluation of nano-sized material toxicity is important. We previously found that nano-sized silica particles with a diameter of 70 nm showed acute liver failure in mice. Here, we performed histological analysis of major organs such as the liver, spleen, lung, kidney, brain and heart in mice, chronically injected with 70-nm silica particles for 4 weeks. Histological analysis revealed hepatic microgranulation and splenic megakaryocyte accumulation in these 70-nm silica particles treated mice, while the kidney, lung, brain and heart remained unaffected. Thus, liver and spleen appear to be the major target organs for toxicity by the chronic administration of the 70-nm silica particles.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

Recent progress in nanotechnology, the act of reducing size from the microscale to the nanoscale, has provided us with dramatic changes in industrial manufacturing and medicine. It also offers many benefits to revolutionize biotechnology, such as synthesis of new drugs with targeted delivery and regenerative medicine [1]. Reducing particle size increases surface area and makes modification of unique physicochemical properties, such as high conductivity, strength, durability, and chemical reactivity possible [2]. Thus, the nanotechnology has led to novel materials and innovations in the industry, bioscience and medicine.

Nanomaterials are already being used in bioscience and medicine, such as electronics, sunscreens, cosmetics and medicine for the purposes of diagnosis, imaging and drug delivery. For example, nano-sized silica particles are intended for the systemic and local delivery of drugs [3]. However, the toxicity of the manufactured nano-sized particles has not been fully evaluated.

We previously found that nano-sized particles with a diameter of 70 nm caused acute liver failure, while micro-sized particles with a diameter of 300 or 1000 nm did not [4]. In this study, we performed histological analysis of chronic toxicity induced by intravenous administration of 70-nm silica particles (SP70) for 4 weeks into the major organs, such as liver, lung, spleen, kidney, brain and heart of mice.

2. Materials and methods

2.1. Materials

Nano-sized silica particles with a diameter of 70 nm were obtained from Micromod Partikeltechnologie GmnH (Rostock, Germany). The surface was not modified. The mean diameters of these particles analyzed by Zetasizer (Sysmex Co., Kobe, Japan) were determined to be 55.7 nm. The particles were spherical and nonporous, and were stored at 25 mg/ml in aqueous suspension. The suspensions were thoroughly dispersed with sonication before use and diluted in water. The dispersion of the particles was confirmed by electron microscopy (data not shown). Reagents used were of research grade.

2.2. Animals

The 8-week-old BLAB/c male mice were purchased from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan), and housed in an environmentally controlled room at 23 ± 1.5 °C with a 12-h light/dark cycle. Mice had access to water and chow (Type MF, Oriental Yeast, Tokyo, Japan) *ad libitum*. Mice were intravenously

Abbreviations: SP70, 70-nm silica particles; HE, hematoxylin–eosin; ALT, alanine aminotransferase; HYP, hydroxyproline.

* Corresponding author. Laboratory of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871, Japan. Tel.: +81 6 6879 8196; fax: +81 6 6879 8199.
E-mail address: masuo@phs.osaka-u.ac.jp (M. Kondoh).

0939-6411/$ - see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2009.03.007

Please cite this article in press as: H. Nishimori et al., Histological analysis of 70-nm silica particles-induced chronic toxicity in mice, Eur. J. Pharm. Biopharm. (2009), doi:10.1016/j.ejpb.2009.03.007

injected with vehicle or the particles twice a week for 4 weeks. On day 3 after the last injection, the mice were sacrificed, and the serum and organs were recovered. The experimental protocols conformed to the ethical guidelines of the Graduate School of Pharmaceutical Sciences, Osaka University.

2.3. Histological analysis

The liver, spleen, lung, kidney, brain and heart were removed and fixed with 4% paraformaldehyde. After sectioning, thin sections of tissues were stained with hematoxylin and eosin for histological observation.

2.4. Biochemical analysis

Serum alanine aminotransferase (ALT) was measured using a commercially available kit according to the manufacturer's protocol (Wako Pure Chemical, Osaka, Japan).

2.5. Hydroxyproline (HYP) assay

Hepatic HYP content was measured by Kivirikko's method with some modification [5]. Briefly, liver tissue was hydrolyzed in 6 M HCl at 110 °C for 24 h. The resultant supernatant was neutralized with 8 N KOH, and then 2 g of KCl and 1 ml of 0.5 M borate buffer were added, followed by a 15-min incubation at room temperature and further incubation for 15 min at 0 °C. Chloramines-T solution was then prepared and added. After additional incubation for 1 h at 0 °C, 2 ml of 3.6 M sodium thiosulfate was added, followed by incubation at 120 °C for 30 min. Next, 3 ml of toluene was added with incubation for a further 20 min at room temperature. After centrifugation, 2 ml of the resultant supernatant was added to Ehrlich's reagent, followed by incubation for 30 min at room temperature. Subsequently, absorbance was measured at 560 nm.

2.6. Statistical analysis

Statistical analysis was performed by Student's *t*-test. The level of significance was set at $p < 0.05$.

3. Results and discussion

We previously found that intravenous administration of SP70 induced liver injury through a single administration [4]. To investigate the chronic toxicity of SP70, 10 or 30 mg/kg of SP70 was intravenously injected into mice twice a week for 4 weeks at which point the livers were not injured or injured by the single injection, respectively [4]. During chronic administration, no significant differences were observed in the body weight between the vehicle and the SP70-treated group (Fig. 1) and no abnormal behaviors were detected (data not shown). Therefore, SP70 treatment did not show apparent toxicity in mice at the low dose.

Next, we performed histological analysis of tissues that are enriched with reticuloendothelial system (RES) such as the liver, spleen, and lungs and non-RES organs such as the heart, kidney and brain. As shown in Fig. 2A and B, treatment with SP70 induces hepatic microgranulation and increases splenic megakaryocyte accumulation. In contrast, the remaining RES organ, the lung, and all the non-RES organs did not show tissue injury with SP70 treatment (Fig. 2B–F). Thus, we examined a serum biochemical marker of liver injury, ALT, to confirm liver injury. SP70 treatment significantly elevated serum ALT levels (Fig. 3A), but a renal injury marker, blood urea nitrogen, was not elevated by these treatments

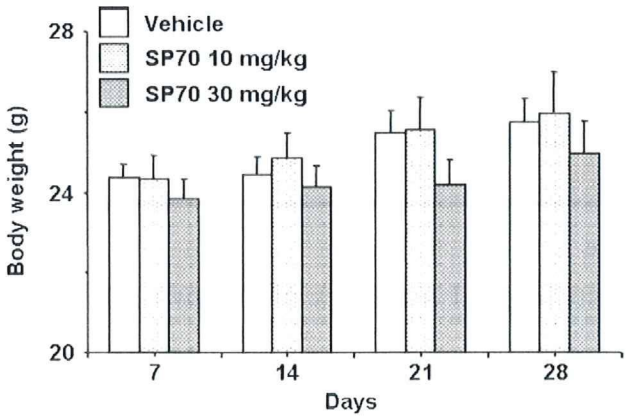

Fig. 1. Body weight changes in mice treated with SP70 for 4 weeks. Mice were intravenously administered SP70 at 0, 10 or 30 mg/kg twice a week for 4 weeks. The body weights of the treated mice were monitored on days 7, 14, 21 and 28. Each point represents mean ± SEM ($n = 5–7$).

(data not shown). Chronic hepatic injury causes liver fibrosis, finally leading to hepatic carcinoma. The chronic treatment with SP70 also elevated a marker of fibrosis, HYP, in the liver (Fig. 3B). Taken together, chronic SP70 treatment appears to injure the liver and spleen.

As innovative materials cover wide fields from industry to life science, nanomaterials have potential to improve the quality and performance of many consumer products as well as medical therapies. Thus, it is very critical in the field of nanotechnology to also assess the risk of nano-sized materials. As the use of nano-sized silica particles in cosmetics and the application in pharmaceutical research, e.g., drug delivery and molecular imaging [3,6] are increasing, we evaluated the toxicity of nano-sized silica particles. We have already found that SP70 causes acute liver injury in mice [4]. In the present study, we evaluate the influence of chronic administration of SP70 for 4 weeks on major organs by histological analysis. As the nano-sized particles are taken into RES organs such as the liver, lung and spleen, we expected that all the RES organs would be injured by chronic SP70 exposure. However, histological abnormalities in the lung were not observed. Kim et al. found that 50-nm silica particles were distributed into all the RES organs, but the amount of the distributed particles into the lung was smaller than that into the liver and spleen [7]. Therefore, the lack of histological abnormalities in the lung may be due to a lower distribution of SP70.

The underlying mechanism for histological injury in the liver and spleen remains to be elucidated. We previously found that the serum levels of inflammatory cytokines (interleukin-6 and tumor necrosis factor-α) were elevated by SP70 [4]. Uptake of SP70 by macrophages in the liver and spleen may cause the release of the cytokines from the macrophage, leading to histological abnormalities. Macrophage receptor with collagenous structure (MARCO), CD204 and CD36 are all macrophage silica particle receptors [8–10]. CD36 is expressed in macrophages of BALB/c mice [10].

In the present study, there is no observation of histological injury in lung, kidney, brain and heart. Regulation of liver and spleen injuries may be critical for the safe application of these nano-sized silica particles. Future analysis is necessary to determine tissue distribution of SP70. Extensive studies are also required to provide the basis for a new class of nanomaterials for drugs, proteins, and gene delivery applications. We are developing materials and methods to control the bio-distribution of these nano-sized silica particles.

Please cite this article in press as: H. Nishimori et al., Histological analysis of 70-nm silica particles-induced chronic toxicity in mice, Eur. J. Pharm. Biopharm. (2009), doi:10.1016/j.ejpb.2009.03.007

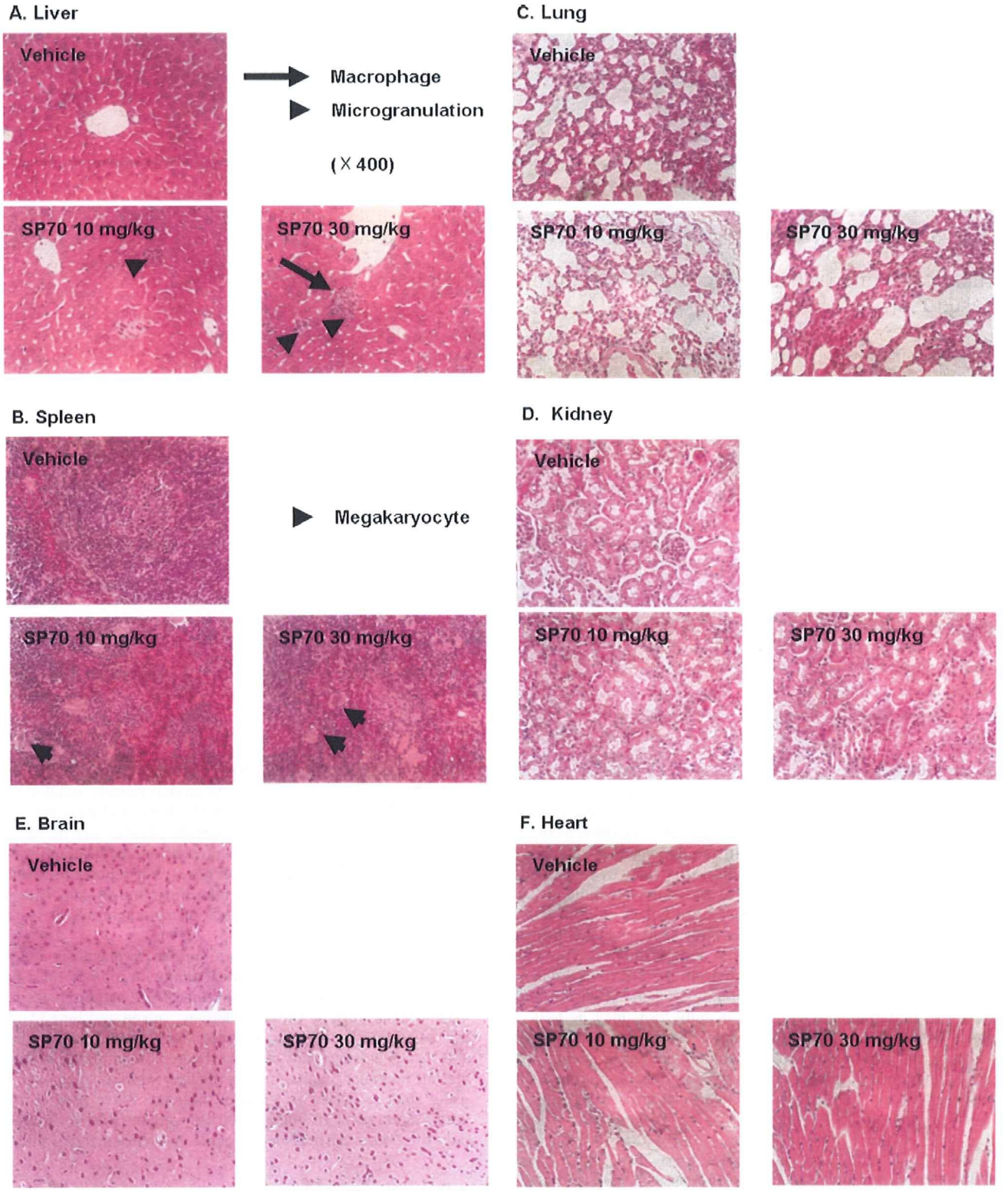

Fig. 2. Histopathological evaluation of the organs from SP70-treated mice. After chronic treatment with SP70 for 4 weeks, liver (A), spleen (B), lung (C), kidney (D), brain (E) and heart (F) were recovered and fixed with paraformaldehyde, followed by staining with hematoxylin and eosin. The arrowheads in (A) and (B) indicate microgranulation in the liver and accumulation of megakaryocyte in the spleen, respectively. The tissue sections were observed under a microscope at 400×. The pictures are representative of at least four independent sections.

Please cite this article in press as: H. Nishimori et al., Histological analysis of 70-nm silica particles-induced chronic toxicity in mice, Eur. J. Pharm. Biopharm. (2009), doi:10.1016/j.ejpb.2009.03.007

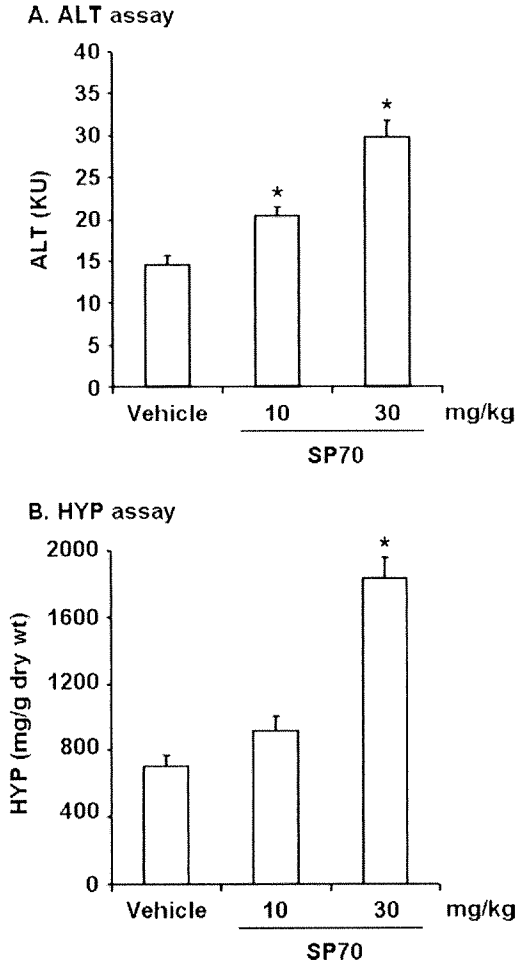

Fig. 3. Biochemical analysis of liver injury. After chronic SP70 treatment for 4 weeks, the serum and liver were collected. Then, the serum alanine aminotransferase (A) and hepatic hydroxyproline (HYP) levels (B) were measured as described in Section 2. Data are shown as mean ± SD ($n = 4$). The results are representative of at least three independent experiments. *Significant difference from the vehicle-treated group ($p < 0.05$).

Acknowledgements

We thank all members of our laboratory for their useful comments and discussion. This study is partly supported by a Grant-in-Aid from the Ministry of Health, Labor, and Welfare of Japan.

References

[1] M.C. Roco, Nanotechnology: convergence with modern biology and medicine, Curr. Opin. Biotechnol. 14 (2003) 337–346.
[2] A. Nel, T. Xia, L. Madler, N. Li, Toxic potential of materials at the nanolevel, Science 311 (2006) 622–627.
[3] M. Vallet-Regi, F. Balas, D. Arcos, Mesoporous materials for drug delivery, Angew. Chem. Int. Ed. Engl. 46 (2007) 7548–7558.
[4] H. Nishimori, M. Kondoh, K. Isoda, S. Tsunoda, Y. Tsutsumi, K. Yagi, Silica nanoparticles as hepatotoxicants, Eur. J. Pharm. Biopharm., in press, doi:10.1016/j.ejpb.2009.02.005.
[5] K.I. Kivirikko, O. Laitinen, D.J. Prockop, Modifications of a specific assay for hydroxyproline in urine, Anal. Biochem. 19 (1967) 249–255.
[6] T.J. Yoon, J.S. Kim, B.G. Kim, K.N. Yu, M.H. Cho, J.K. Lee, Multifunctional nanoparticles possessing a "magnetic motor effect" for drug or gene delivery, Angew. Chem. Int. Ed. Engl. 44 (2005) 1068–1071.
[7] J.S. Kim, T.J. Yoon, K.N. Yu, B.G. Kim, S.J. Park, H.W. Kim, K.H. Lee, S.B. Park, J.K. Lee, M.H. Cho, Toxicity and tissue distribution of magnetic nanoparticles in mice, Toxicol. Sci. 89 (2006) 338–347.
[8] L. Kobzik, Lung macrophage uptake of unopsonized environmental particulates. Role of scavenger-type receptors, J. Immunol. 155 (1995) 367–376.
[9] A. Palecanda, J. Paulauskis, E. Al-Mutairi, A. Imrich, G. Qin, H. Suzuki, T. Kodama, K. Tryggvason, H. Koziel, L. Kobzik, Role of the scavenger receptor MARCO in alveolar macrophage binding of unopsonized environmental particles, J. Exp. Med. 189 (1999) 1497–1506.
[10] R.F. Hamilton Jr., S.A. Thakur, J.K. Mayfair, A. Holian, MARCO mediates silica uptake and toxicity in alveolar macrophages from C57BL/6 mice, J. Biol. Chem. 281 (2006) 34218–34226.

Please cite this article in press as: H. Nishimori et al., Histological analysis of 70-nm silica particles-induced chronic toxicity in mice, Eur. J. Pharm. Biopharm. (2009), doi:10.1016/j.ejpb.2009.03.007

European Journal of Pharmaceutics and Biopharmaceutics xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Silica nanoparticles as hepatotoxicants

Hikaru Nishimori [a], Masuo Kondoh [a,*], Katsuhiro Isoda [a], Shin-ichi Tsunoda [b,c], Yasuo Tsutsumi [b,c,d], Kiyohito Yagi [a]

[a] Laboratory of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan
[b] Laboratory of Pharmaceutical Proteomics, National Institute of Biomedical Innovation, Osaka, Japan
[c] The Center for Advanced Medicinal Engineering and Informatics, Osaka University, Osaka, Japan
[d] Laboratory of Toxicology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan

ARTICLE INFO

Article history:
Received 16 October 2008
Accepted in revised form 9 February 2009
Available online xxxx

Keywords:
Silica particle
Nano-size particle
Liver injury

ABSTRACT

Nano-size materials are increasingly used in cosmetics, diagnosis, imaging and drug delivery, but the toxicity of the nano-size materials has never been fully investigated. Here, we investigated the relationship between particle size and toxicity using silica particles with diameters of 70, 300 and 1000 nm (SP70, SP300, and SP1000) as a model material. To evaluate acute toxicity, we first performed histological analysis of liver, spleen, kidney and lung by intravenous administration of silica particles. SP70-induced liver injury at 30 mg/kg body weight, while SP300 or 1000 had no effect even at 100 mg/kg. Administration of SP70 dose-dependently increased serum markers of liver injury, serum aminotransferase and inflammatory cytokines. Repeated administration of SP70 twice a week for 4 weeks, even at 10 mg/kg, caused hepatic fibrosis. Taken together, nano-size materials may be hepatotoxic, and these findings will be useful for future development in nanotechnology-based drug delivery system.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

The recent development of technology for reducing material size has provided innovative nanomaterials. Nanomaterials are engineered structures with at least one dimension of 100 nm or less, and have unique physicochemical properties with regard to size, chemical composition, surface structure, solubility, shape and aggregation. Nanomaterials have been widely used in microelectronics, catalysts, ultra-sensitive molecular sensing and imaging probes, pharmaceutical agents and cosmetics. Thus, the development of reduced particle size from the macro to the nanoscale provides benefits to a range of industrial and scientific fields. However, materials that are inert in bulk form may be toxic in nano-size forms, and it is thus essential to understand the biological activities and potential toxicity of nanomaterials [1–3].

The influence of inhalation of nanomaterials on human health has been widely investigated. Occupational exposure to quartz, mineral dust particles and asbestos induce inflammation, fibrosis and cytotoxicity in the lung [3]. In animal models, inhaled nanoparticles do not locally remain in the lung, and pass into blood flow, resulting in distribution to distant organs, such as the liver, kidney, brain and heart [4–7]. Moreover, biomedical applications for diagnosis and therapeutic purposes will require intravenous, subcutaneous or intramuscular administration [8–10]. Thus, it is necessary to confirm the influence of nanomaterials in systemic flow on various organs.

Silica nanoparticles have been applied to diagnosis and drug delivery [4,11], and intraperitoneal administration of silica nanoparticles results in the biodistribution of the nanoparticles to diverse organs, such as the liver, kidney, spleen and lung [4]. Both micro- and nano-size silica particles are also commercially available. In the present study, we investigated the influence of nanomaterials on major organs, such as the liver, kidney, spleen and lung using silica particles as a model material. When silica particles with a diameter of 70, 300 or 1000 nm were intravenously injected, only the 70-nm particles led to acute and chronic liver injury.

2. Materials and methods

2.1. Materials

Silica nanoparticles with a diameter of 70, 300 or 1000 nm were purchased from Micromod Partikeltechnologie GmbH (Rostock,

Abbreviations: SP70, 70 nm silica particles; SP300, 300 nm silica particles; SP1000, 1000 nm silica particles; ALT, aminotransferase; BUN, blood urea nitrogen; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; $GdCl_3$, gadolinium chloride; CPA, cyclophosphamide; LSEC, liver sinusoidal endothelial cells; MARCO, macrophage receptor with collagenous structure.

* Corresponding author. Laboratory of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 5650871, Japan. Tel.: +81 6 6879 8196; fax: +81 6 6879 8199.
E-mail address: masuo@phs.osaka-u.ac.jp (M. Kondoh).

0939-6411/$ - see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2009.02.005

Please cite this article in press as: H. Nishimori et al., Silica nanoparticles as hepatotoxicants, Eur. J. Pharm. Biopharm. (2009), doi:10.1016/j.ejpb.2009.02.005