breast tumor-induced osteolysis [12]. Herein, we used gene expression profiles from our mouse model and Connectivity Map database to find therapeutic agents that target the TB interface, rather than a given pathway.

The advantage of Connectivity Map database is that it can predict potential therapeutic agents based solely on gene signatures [39]. In the current study, our query of Connectivity Map database with the TB signature flagged cyclopenthiazide in the MCF7 cell line (Figure 4D). This analysis suggests that cyclopenthiazide has the potential to inhibit the establishment of breast cancer cells at TB interface.

Thiazides comprise a class of diuretic agents (of which cyclopenthiazide is a member) that are traditionally used to treat hypertension and edema [64]. Although thiazides have not been widely viewed as therapeutic agents for bone metastasis, reports abound noting that treatment of hypertension using thiazides has the beneficial side effect of strengthening bone [65-69]. Furthermore, Devorak *et al.* have demonstrated that the bone strengthening activity of thiazides results from their direct action on OCPs, where thiazide analogs are able to directly induce osteoblast differentiation [70]. These data suggest that cyclopenthiazide may be a useful agent against osteoclastic bone metastasis. Future efforts are aimed at validating this prediction in the osteolytic mouse model. This study serves as an example of how mouse breast cancer-specific osteolytic models and gene expression analysis can be used to identify treatment strategies for human disease.

Conclusions

In summary, we have demonstrated that the TB microenvironment in our mouse model of osteolytic breast cancer metastasis is highly similar to that of human breast cancer-to-bone metastases. Furthermore, gene expression profile analysis of tumors from this model: (i) identified a TB interface specific gene signature; (ii) revealed signaling pathways that were differentially activated at the TB interface and TA area; (iii) demonstrated a role for osteoclasts in metastatic osteolysis; and (iv) predicted a novel therapeutic agent that specifically targets the TB interface. These data clearly demonstrate that this mouse model can be used to study the cellular and molecular mechanisms driving human breast cancer-to-bone metastasis and osteolysis. Moreover, this model also provides a powerful preclinical setting to test thiazides and other therapeutic agents that specifically target breast cancer osteolysis.

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Authors' contributions

AS conceived and developed the concept, performed all the analyses and wrote the manuscript. MF performed the animal experiments. CAL contributed to the scientific content. CAL and WJG helped with editing of the manuscript. MF and RKS conceived the idea for the development of the animal model. All authors read and approved the final manuscript.

Competing interests

WJG is currently an employee of Genomic Health.

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—Review—

ナノマテリアルの慢性影響研究の重要性

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Importance of Researches on Chronic Effects by Manufactured Nanomaterials

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Manufactured nanomaterials are the most important substances for the nanotechnology. The nanomaterials possess different physico-chemical properties from bulk materials. The new properties may lead to biologically beneficial effects and/or adverse effects. However, there are no standardized evaluation methods at present. Some domestic research projects and international OECD programs are ongoing, in order to share the health impact information of nanomaterails or to standardize the evaluation methods. From 2005, our institutes have been conducting the research on the establishment of health risk assessment methodology of manufactured nanomaterials. In the course of the research project, we revealed that the nanomaterials were competent to cause chronic effects, by analyzing the intraperitoneal administration studies and carcinogenic promotion studies. These studies suggested that even aggregated nanomaterials were crumbled into nano-sized particles inside the body during the long-term, and the particles were transferred to other organs. Also investigations of the toxicokinetic properties of nanomaterials after exposure are important to predict the chronically targeted tissues. The long lasting particles/fibers in the particular tissues may cause chronic adverse effects. Therefore, focusing on the toxicological characterization of chronic effects was considered to be most appropriate approach for establishing the risk assessment methods of nanomaterials.

Key words——chronic toxicity; multi-wall carbon nanotube (MWCNT); fullerene

1. はじめに

近年，ナノテクノロジーの中心的な役割を担う物質としての産業用ナノマテリアルは，急速にその種類や生産量が増加しつつあるところであるが，新たに期待されているナノマテリアルの物理化学特性については，有効的な生理活性等に使用され得る特性を持つ反面，ヒト健康影響に対する懸念についても検証されるべきであると考えられている．つまり，ナノマテリアルを用いた技術や製品を社会的に受容するためには，安全性の検証を行うことが不可欠であると思われる．しかし，従来の一般的な化学物質とは異なる物理化学的特性は，その毒性評価においても従来とは異なる考え方を取り入れることも必要とされている．それゆえ，ナノマテリアルの特性を考慮した有害性評価手法の開発が急務となっている．また，国際的な枠組みにおいても，ナノマテリアルの安全性確認は，重要な問題として認識されており，OECD や ISO 等を中心として評価手法の国際的標準化に向けた取り組みが進行しているところでもある．本稿では，ナノマテリアルの安全性評価

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本総説は，日本薬学会第 130 年会シンポジウム S18 で発表したものを中心に記述したものである．

の確立に向けたこれらの取り組みに貢献してきたわれわれの研究成果の一部と，それらの研究結果から帰納的に導き出された慢性影響評価研究の重要性について論ずる．

2. ナノマテリアルのリスク評価法の確立における課題

一般的に，化学物質の健康影響評価（リスクアセスメント）の基本的なフレームは，有害性評価と曝露評価，及び各々の評価内容を比較・統合化する過程のリスク判定のステップから成り立っている．この基本的なフレーム自体は，ナノマテリアルの健康影響評価に適用できるものであると考えられる．[1-5] しかし，ナノマテリアルに特徴的な新たな物理化学的性質，特にサイズが生体内高分子と近いことや，高い表面活性のために凝集し易い性質を考慮すると，よりサイズの大きい通常のバルク化合物や完全に溶解した単一分子化合物とは，生体内挙動が異なることが予想され，同じ化学組成の化合物であってもその毒性発現部位や発現様式は異なることが予想される．つまり，体内動態［吸収 absorption，分布 distribution，代謝 metabolism，排泄 excretion (ADME)］情報は，一般の化学物質より重要な意味を持つと考えられる．

そこで，生体内での挙動を把握するためには，生体試料中で検出，同定・定量できる方法を確立しなくてはならない．一般にナノマテリアルの開発段階において，その性質を把握するための物理化学的測定法も同時に開発されているはずであるが，それらの手法は生体試料中に存在するナノマテリアルにそのまま適用できないことも多い．さらに，機器分析法による生体試料中での検出や定量が可能になったとしても，生体内で実際にナノの状態で存在しているのか，あるいは再凝集などはしていないかなど，標的組織における最終的な生体内反応に影響を及ぼすと考えられる実際のナノマテリアルの存在状態を把握するためには，最終的には，組織標本の電子顕微鏡などによる確認が必要となる．

一方，体内動態に影響を与える因子として，投与法を検討する必要もある．単独では凝集し易いナノマテリアルをそのまま曝露するということは，物理的に巨大となった粒子は体への吸収性が低く，ナノマテリアル自体の体内動態や懸念される有害性を検出することが困難になると考えられるためである．そのために曝露実験時におけるナノマテリアルの分散手法の開発が必要となる．職業曝露などの比較的大量のナノマテリアル曝露の安全性を評価するという観点からは，凝集したままの曝露にも意義があるかもしれないが，製品中への混入や環境中への排出を経由した，分散された曝露も想定されることは考慮すべきであると考えられる．

Figure 1 は，凝集したナノマテリアルが，生体に取り込まれた場合に想定される体内動態を模式図化したものである．ナノマテリアルの使用用途にも依存するが，製品中のナノマテリアルはポリマー等の他の高分子化合物等と混合された状態，あるいはナノマテリアルだけが単独で製品から解離していく状態を考慮しても，この凝集性のために，大きな粒子として曝露する可能性が高いものと想定される．急性的には，このサイズの大きくなった物質は生体に取り込まれることはほとんどなく，局所的な刺激を起こすような変化を除いては，生体内で有害性が惹起される可能性は低いものと考えられる．しかし，仮に凝集したナノマテリアルが長期間に渡って，吸収部位である肺胞や消化管，損傷皮膚などの局所に滞留したり，慢性的に曝露したりするケースを想定すると，時間経過とともに小さくなった凝集体の粒子を除去するために，マクロファージなどの食細胞による取り込みや，表面活性の高いナノマテリアル分子と生体成分との結合作用による侵食作用により，生体に少しずつ取り込まれることが想定される．もしも生体内に取り込まれたナノマテリアルと生体内成分との結合性が高い場合には，容易に生体外に排出されることはなく，特定の組織等へ蓄積し易くなり，慢性影響の可能性を検討する必要が出てくると想定できる．

3. 国立医薬品食品衛生研究所における取り組みの成果の概要

以上のナノマテリアル固有の検討課題を考慮して，われわれは 2005 年より厚生労働科学研究の化学物質リスク研究事業の枠組みの中で，ナノマテリアルの健康影響評価手法の開発に係わる研究を推し進めてきたところである．われわれは，これらの検討課題を解決するために，Fig. 2 に示すように 4 つの項目を中心に研究を行ってきた．これらの項目の中で，*in vivo* 研究については，比較的研究初期の段階から中心的に取り組んできた．その中で，繊維

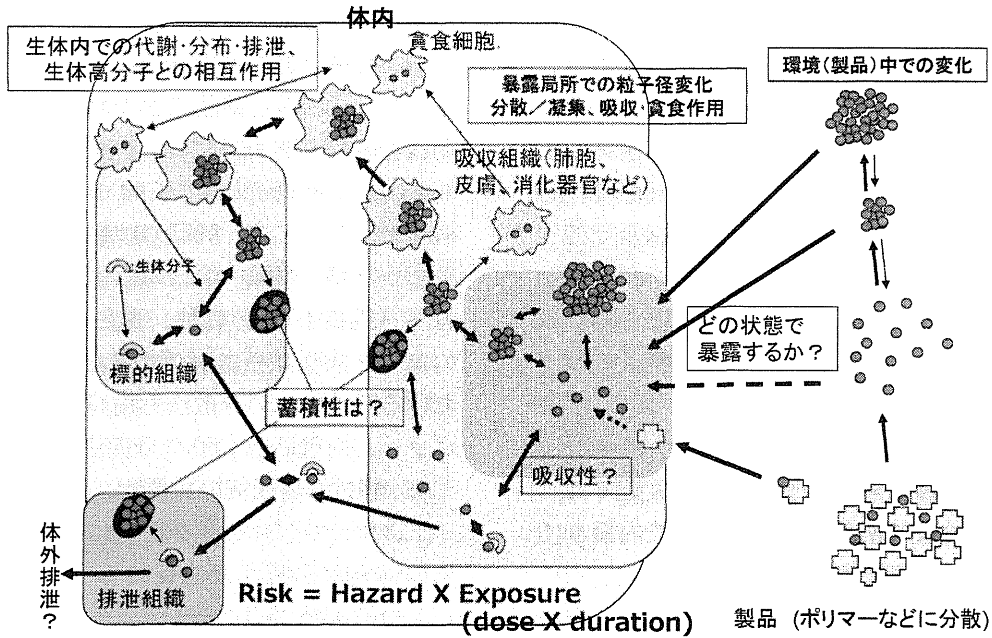


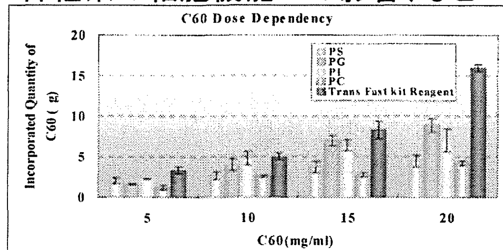
Fig. 1. The Estimated ADME Schema of Nanomaterials

***in vivo*試験法研究**
MWCNTのP53ヘテロ欠失マウスへのi.p.投与による中皮腫誘発性を確認
バイオマーカとしてマウスのメソセリン抗体の作成
一方、C60の腹腔内投与による慢性的影響として腎臓への影響を示唆
TiO_2と C60の気管内投与による発がんプロモーション作用の示唆

吸入試験法研究
MWCNTのミスト暴露システムを開発
気管内投与時の分散性依存の発現様式差異を確認
リポソーム分散C60による気管内投与法を開発。

暴露測定法／動態解析研究
生体試料でのC60の定量的検出法との確立
静注後のC60の組織からの経時的消失検討
気管内投与後のMWCNTの肺及び肝臓での検出

***in vitro*試験法研究**
細胞培養系でのリポソーム等を用いた分散法の確立
→C60やTiO_2の遺伝毒性、細胞透過性、
神経系の細胞機能への影響、などへの適用



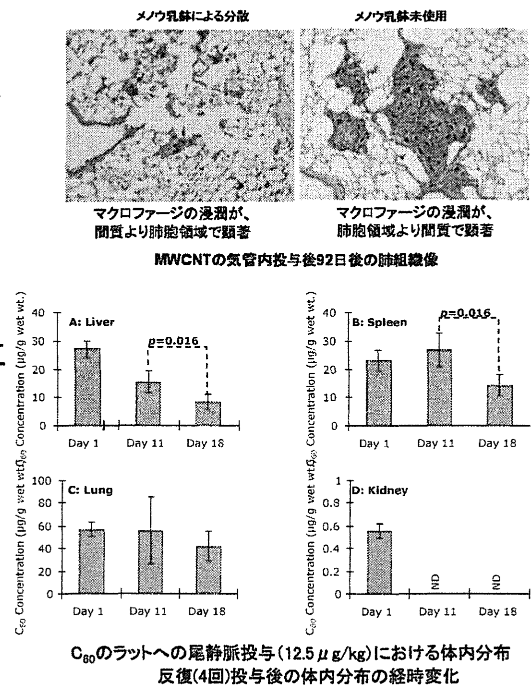


Fig. 2. The Overall Results of NIHS Projects for Nanomaterial Safety

長の長いタイプの多層型カーボンナノチューブ（MWCNT）が，中皮腫を誘発する可能性を持つことを確認した.[6] 上記の体内動態の重要性を考慮した概念からは，吸収性や体内分布について検証したのちに，慢性影響の可能性を検討することが論理的であるが，研究開始当時から，大量生産可能であった，酸化チタン（TiO_2）やフラーレン（C60），MWCNT については，*in vivo* の慢性影響を先行して検討しておくべきであると判断した．特にその形状がアスベストに似ていた MWCNT については，吸入曝露による有害影響が懸念されたが，MWCNT についての吸入曝露法が確立していない段階では，アスベストでも検証に使用されていた腹腔内投与による中皮腫誘発試験を行うこととした.

われわれの最初の実験は，アスベストで中皮腫の誘発時期が早くなることが知られている p53 ヘテロノックアウトマウスへの腹腔内へ 3 mg/mouse という高用量を投与することによって確認されたものであり，動物種の特異性や投与量の多さについて異論も指摘された．しかしその後の研究で，野生型の動物種である F344 ラットに対しても，同じ MWCNT が中皮腫の誘発作用を持つことが確認された[7]ほか，投与量を 1000 分の 1 にまで少なくした実験においても中皮腫の起きることが示されている（投稿中）.

酸化チタンについては，雌ラットへの吸入曝露により発がん性のあることが示されているが，ナノサイズ化による発がん性の検証のために，気管内投与による肺がんのプロモーション作用の検討を行った．その結果，酸化チタンは，肺腺腫や乳腺腫に対してプロモーション作用を示し，その作用は，マクロファージから放出される炎症性因子である MIP1α を介したものであることが示唆された.[8] 現在 C60 や MWCNT を用いたプロモーション作用の検討が進行中である.

一方，曝露手法の開発においては，ミスト法や粉体法による MWCNT の吸入曝露システムの開発研究を進めているが，より簡易な手法として気管内投与のための適切な分散法の検討を行った．その結果，分散法の違いが肺の有害性発現様式に違いを引き起こすことを確認した.[9]

体内動態解析のために，生体試料中の C60 や TiO_2 の分析手法の開発や改良を行い，経口投与や気管内投与による体内吸収性について検討を行っている．現在のところ投与部位である消化管や肺以外で有意な検出量を確認できておらず，感度の向上に向けた研究を進めている．しかし，体内への吸収を前提にした解析として，C60 の静脈内投与による解析を行ったところ，肝臓や脾臓，肺などへの分布を確認したが，腎臓への分布は極めて低いことが示された（投稿中)．その他，遺伝毒性や標的臓器などの毒性をスクリーニングするための *in vitro* 試験における培地等への分散法も検討対象としており，リポソームを用いた C60 の分散法を確立した.

4. 慢性影響研究の重要性

ナノマテリアルの生体影響に関する情報はここ数年の活発な研究状況を反映して多くなりつつあるが，慢性影響に関する報告は依然その数が少ない状況である．一般の化学物質の有害性評価の常套手段として，変異原性試験や短期試験から情報を収集していくことは，必要なステップであり，OECD におけるナノマテリアル作業グループの活動におけるスポンサーシッププログラムにおいても，加盟各国からの毒性試験情報として，短期試験を中心に収集されてきている．われわれの研究グループにおいても，これらの枠組みに対して，短期的な試験情報を中心に提供し始めている段階である．しかし MWCNT に関しては，研究初期から，短期毒性より長期毒性の方が懸念の強いことが，物性等の情報から推測されたところでもあり，その推定に基づいて，腹腔内投与の研究を最初にスタートさせた．腹腔内投与は，リスク評価の観点からは，曝露経路（吸入曝露）に伴う定量的な評価に問題のあるところであるが，最近の注目すべき研究として，分散剤で分散させた MWCNT（最高 80 μg まで）をマウスに吸引させた研究や，MWCNT: 30 mg/m^3 をマウスに単回吸入曝露した研究において，曝露後 7–8 週間目に MWCNT が胸膜に到達していたことが報告されている.[10,11] これらの研究結果は高用量の曝露による短期間の結果ではあるが，呼吸器を経由した曝露においても MWCNT は胸膜（中皮）まで到達することを示唆しており，われわれの腹腔内投与による結果と合わせると，リスク評価の上でも重要な知見であると考えられる.

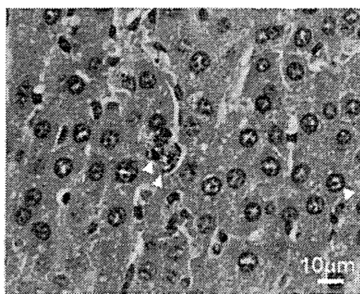
これらの腹腔内投与による中皮腫誘発能は，繊維状粒子による催腫瘍性のみを検出する系であり，短

いタイプやその他様々な形状の MWCNT における慢性毒性は別途検証する必要性がある．実際，われわれの行った腹腔内投与試験では，小さいサイズのナノチューブ繊維を含んだ細胞が腹膜の病変部のみならず，肝類洞内，又は肝葉間や腸間膜リンパ節の中にも認められ，体内に再分布することが示唆された（Fig. 3）.[6] さらに，SWCNT をマウスへ咽頭吸引させた実験では，一過性の急性症状の後に，炎症性細胞浸潤を伴わない間質の繊維化が認められている.[12] また，ApoE ノックアウトマウスを用いた実験では，タンパクカルボニル化活性の変化を伴うミトコンドリア DNA 障害と，アテローム性動脈硬化症の進行を増強することが示された.[13] MWCNT に関しても，マウスに MWCNT（200–400 μg）を気管内滴下した実験では，一過性の肺の炎症反応に加え，投与量に依存した血小板の活性化と凝固作用の活性化の促進が示唆されている.[14] また，MWCNT や SWCNT の気管内投与や経鼻投与により，アレルギー反応の増強反応が報告されている.[15-17] これらの結果が，カーボンナノチューブが直接体内循環に侵入した結果であるか，免疫細胞との接触を介した反応であるかを区別することは難しいが，曝露局所に留まらない全身作用の可能性を示している．われわれの酸化チタンの気管内投与による発がんプロモーション作用が，炎症因子により介在されたことは，これらの知見と同様の作用様式を示すものととらえることもできる．

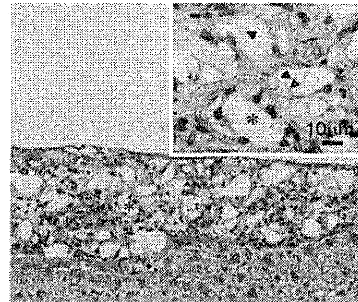
以上の知見は，短期の試験だけでは検証することは困難であり，ナノマテリアルの有害性を確認するためには，長期の体内動態予測や慢性影響に関する研究が，重要なステップであることを示している．Figure 4 にスクリーニング試験や確定試験を開発するための手順についてまとめた．通常の化学物質については，その長い歴史の中で明らかとなった有害性に対して，それぞれの毒性発現様式に応じてスクリーニング試験が開発され，現在まで運用されてきている．特に変異原性試験は発がん性を予測する試験としての重要な役割を担っている．しかし，現時点ではナノマテリアルによる有害性影響が，これまでの研究経験の中で明らかとなった影響だけに留まるのかについては，まだ誰も判定できない状況である．これまでの一般化学物質に対応する有害性とスクリーニング試験を活用して進めていくと同時に，未知の影響を見極める最初のステップとして，少な

腹腔内投与によるナノサイズ粒子の体内再分布

肝臓内類洞 (MWCNT)



腹膜の漿膜 (fullerene)



A. Takagi et al., J. Toxicol. Scie., **33**,105-116. (2008)

SWCNTやMWCNTによる全身性影響の示唆

- アテローム性動脈硬化症の進行の増強の可能性（ApoE$^{-/-}$マウス）
  Z. Li *et al.*, *Environmental health perspectives*. **115**, 377-382 (2007)
- 血小板の活性化と凝固作用の活性化（MWCNT気管内滴下）
  A. Nemmar *et al.*, *J. Thrombosis, Haemostasis* **5:** 1217-1226 (2007)
- アレルギー反応の増強（ MWCNT・SWCNT、気管内・経鼻投与）
  E.J. Park *et al.*, *Toxicology*. **259**, 113-21 (2009)
  U.C. Nygaard *et al.*, *Toxicol Sci.* **109**, 113-23 (2009)
  K. Inoue *et al.*, *Toxicol Appl Pharmacol*. **237**, 306-16 (2009)

Fig. 3.　The Suggestive Evidences for Systemic Toxicites by Nanomaterials

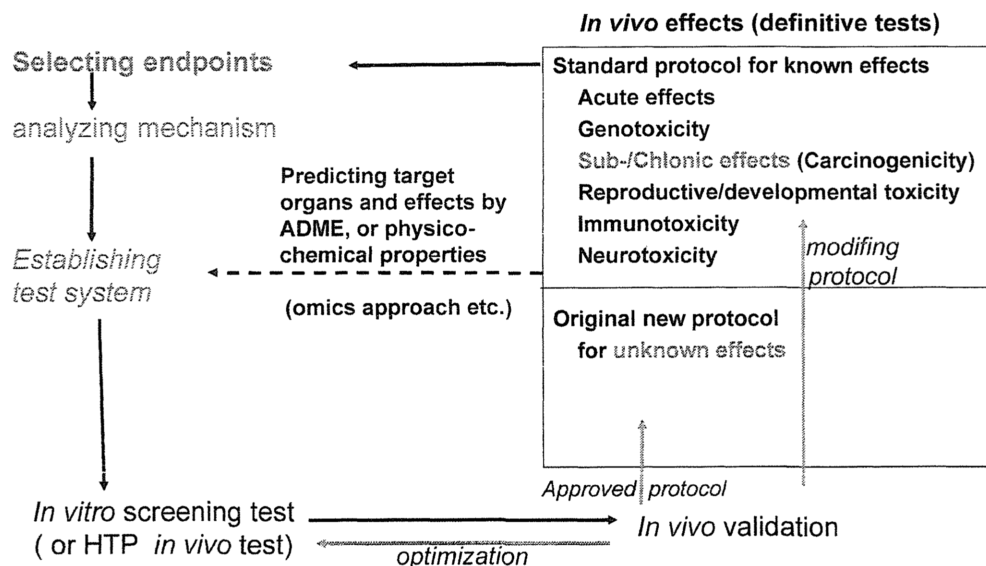


Fig. 4. The Schematic Development of Screening Tests and Definitive Tests

くとも代表的なナノマテリアルによる *in vivo* の慢性影響研究や，その影響を推定するためのナノマテリアルと生体成分との分子レベルでの相互作用や体内残留性様式の解析を進めていくべきであると考えられる．

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Original Article

Lack of promoting effect of titanium dioxide particles on chemically-induced skin carcinogenesis in rats and mice

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ABSTRACT — Nano-sized titanium dioxide particles (TiO_2) are widely used in cosmetics, sunscreens and food additives. We previously reported that topical application of non-coated rutile type TiO_2 did not exhibit a promoting effect on ultraviolet B-initiated skin carcinogenesis in rats, and that this was likely due to lack of penetration of TiO_2 into the epidermis. In the present study, we examined the promoting effect of silicone coated TiO_2 ($sTiO_2$) suspended in silicone oil and non-coated TiO_2 ($ncTiO_2$) suspended in Pentalan 408 on a two-stage skin chemical carcinogenesis model: $sTiO_2$ suspended in silicon oil forms smaller particles than $ncTiO_2$ suspended in Pentalan because of the smaller sizes of aggregates formed. The model used skin carcinogenesis-sensitive human c-Ha-ras proto-oncogene transgenic mice (rasH2) and rats (Hras128) and their wild-type counterparts and CD-1 mice to test the effects of topical application of TiO_2. Animals were initially treated with a single dose of 7,12-dimethylbenz[a]anthracene (DMBA) and then with 0, 10, or 20 mg $sTiO_2$ (mice) or 0, 50, or 100 mg $ncTiO_2$ (rats). The incidence and multiplicity of skin tumors (squamous cell papilloma and carcinoma) did not increase over DMBA alone controls in skin carcinogenesis-sensitive mice or rats or wild-type animals. Analysis of rat skin indicated that $sTiO_2$ and $ncTiO_2$ did not penetrate though either healthy or damaged skin. Furthermore $sTiO_2$ did not penetrate an *in vitro* human epidermis model. Our results indicate that treatment with $sTiO_2$ or $ncTiO_2$ did not promote skin carcinogenesis in mice or rats, probably due to lack of penetration through the epidermis.

Key words: Nano-size TiO_2, Skin carcinogenesis, Hras, Rat, Mouse

INTRODUCTION

Nano-sized titanium dioxide (TiO_2) particles are used in sunscreen formulations to protect against skin lesions caused by exposure to UV light (Gelis *et al.*, 2003; Rouabhia *et al.*, 2002; Suzuki, 1987). Nano and larger scale titanium dioxide particles are known to be carcinogenic to the rat lung (Baan *et al.*, 2006; Baan, 2007). Recently, we demonstrated a promoting effect on rat lung carcinogenesis by nano-size TiO_2 particles administered by a novel intrapulmonary spraying method (Xu *et al.*, 2010). The mechanism of promotion of lung carcinogenesis involved the induction of MIP1α protein expression by $ncTiO_2$-laden alveolar macrophages (Xu *et al.*, 2010).

We also examined the carcinogenic effect of TiO_2 (mean manufacturer's particulate diameter of 20 nm) on the skin in a UVB-initiated two-stage rat carcinogenesis model and found that topical application of TiO_2 did not promote skin carcinogenesis in this model. This result is probably due to the inability of TiO_2 to penetrate through

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the epidermis and reach the underlying tissue (Xu *et al.*, 2011). This speculation is consistent with a report by Newman *et al.* (2009) demonstrating an absence of penetration of TiO_2 through the epidermis and hair follicles (Newman *et al.*, 2009). On the other hand, Wu *et al.* (2009) reported that TiO_2 (4 nm and 60 nm length) could penetrate through the stratum corneum (SC) and become located in the deep layer of the epidermis after being topically applied to pig ear for 30 days (Wu *et al.*, 2009). These inconsistent observations may be due to differences in particle size and the animals used.

The skin is histologically composed of the SC, epidermis, dermis and the subcutaneous tissue. The SC is the rate-limiting barrier against exposure to various exogenous chemical and physical agents (Schaefer *et al.*, 2003). For solid materials, including nano-sized particles, to cause inflammatory lesions, they need to penetrate the SC to interact with macrophages and other inflammatory leucocytes. Long-term activation of inflammatory leukocytes has the potential to cause skin carcinogenesis. Thus, the potential skin-carcinogenicity of TiO_2 is dependent on its size and ability to penetrate through the SC.

The surface of the TiO_2 used in cosmetics is usually coated with aluminum oxide or silicone oils to prevent aggregate formation and to enhance dispersal (Nohynek *et al.*, 2008). The particle size of TiO_2 suspended in silicone oils is known to be smaller than that of non-coated TiO_2 suspensions (Senzui *et al.*, 2010; also compare Fig. 1E with Fig. 1F). In our previous study, we showed that rutile type non-coated TiO_2 ($ncTiO_2$) did not penetrate the epidermal tissue and thus did not cause promotion of chemically-induced skin carcinogenesis. In the present study, we used rutile type TiO_2 coated with silicone ($sTiO_2$) suspended in silicon oil to minimize aggregation and improve the penetrating ability of the particles.

The ability of $sTiO_2$ suspended in silicone oil and $ncTiO_2$ suspended in Pentalan408 to promote skin carcinogenesis was examined using the 7,12-dimethylbenz[a] anthracene (DMBA)-initiated skin carcinogenesis model employing skin carcinogenesis-sensitive animals and their wild-type counterparts as the test animals. The rasH2 mouse carries a human c-Ha-ras proto-oncogene and is highly susceptible to chemically induced skin carcinogenesis (Muto *et al.*, 2006). The Hras128 rat also carries a human c-Ha-ras proto-oncogene and highly susceptible to chemically induced skin carcinogenesis (Park *et al.*, 2004).

In addition to the animal models, we also used an *in vitro* model to examine $sTiO_2$ particle penetration into skin. Unlike animal skin, the *in vitro* model does not have hair follicles, allowing direct examination of the ability of $sTiO_2$ particles to penetrate through a layer of human skin epidermal keratinocytes.

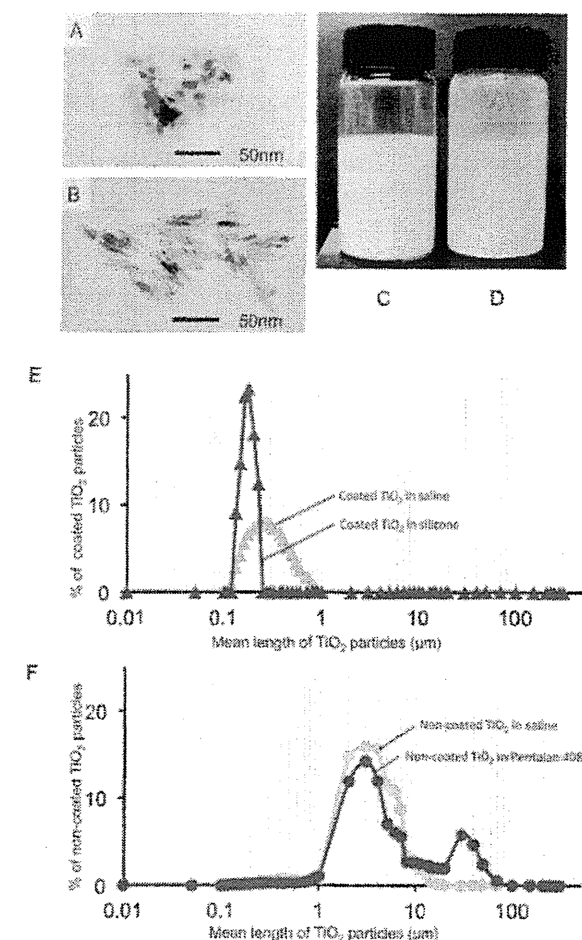

Fig. 1. Physicochemical features of $sTiO_2$ / $ncTiO_2$. $sTiO_2$ particles were round to oval in shape (A), and $ncTiO_2$ particles were club shaped (B). $sTiO_2$ particles remained evenly dispersed in silicone solution (C) while $ncTiO_2$ particles formed a white sediment at the bottom of the bottle 3 days after preparation (D). Size distribution of $sTiO_2$ suspended in saline (gray triangles) and in silicone (black triangles) (E). Size distribution of $ncTiO_2$ suspended in saline (gray circles) and in Pentalan 408 (black circles) (F). % of TiO_2 particles (Y axis) was calculated as the ratio of TiO_2 particles of a particular mean length/total particles examined.

Table 1. TiO_2 materials and animal strains used in this study

Coating status of TiO_2	Size	Concentrartion of TiO_2 (mg/ml)	Suspended in	Skin assay (Carcinogen) Strain (or *in vitro* system)
Coated (rutile type)	35 nm	50, 100	Silicone	Carcinogenesis (DMBA) rasH2 mouse, C57BL mouse
		100, 200	Silicone	Penetration LabCyte EPI-MODEL
Non-coated (rutile type)	20 nm	100, 200	Pentalan 408	Carcinogenesis (DMBA) Hras128 rat, Sprague-Dawley rat
		50, 100	Pentalan 408	Carcinogenesis (DMBA) CD1 mouse
		200	Pentalan 408	Penetration Sprague-Dawley rat

MATERIALS AND METHODS

Animals

Male rasH2 mice and Hras128 rats, known to be highly sensitive to chemically induced skin carcinogenesis (Muto *et al.*, 2006; Park *et al.*, 2004), and their wild-type counterparts, CB6F1 mice and SD rats, were purchased from CLEA Japan Co., Ltd. (Tokyo, Japan). To confirm the results, CD-1 mice, which are frequently used in skin carcinogenesis studies, were also included in this series of studies. The animals were housed in the animal center of Nagoya City University Medical School, maintained on a 12 hr light-dark cycle and received Oriental MF basal diet (Oriental Yeast Co., Tokyo, Japan) and water *ad libitum*. All animals were kept for 1 week for acclimation. The experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals, and the study protocol was approved by the Institutional Animal Care and Use Committee of Nagoya City University Medical School.

Preparation of suspensions of titanium dioxide (TiO_2) and size analysis

$sTiO_2$ particles (silicone coated, mean manufacturer's particulate diameter of 35 nm) and $ncTiO_2$ particles (rutile type, mean manufacturer's particulate diameter of 20 nm) were provided by Japan Cosmetic Association, Tokyo, Japan. Size, coating, dose and suspension vehicles and animals used are summarized in Table 1. The size distribution of $sTiO_2$ suspended in silicone oil (cyclopentasiloxane, KF-995, Shin-Etsu Chemicals Co., Tokyo, Japan) or in saline and $ncTiO_2$ particles suspended in Pentalan 408 (pentaerythritol tetraethylhexanoate, CAS7299-99-2, Nikko Chemicals Co., Tokyo, Japan) or in saline was determined by a Particle Size Distribution Analyzer (Shimadzu Techno-Research Inc., Kyoto, Japan). The shape of suspended $sTiO_2$ and $ncTiO_2$ was observed by transmission electron microscopy (JEOL Co. Ltd., Tokyo, Japan). Freshly made suspensions were sonicated for 30 min, and suspensions were sonicated again for 30 min just prior to use.

Experimental design

Skin carcinogenesis study of silicone coated TiO_2 ($sTiO_2$) using rasH2 mice

The back skin of 7-week-old female rasH2 mice (60 mice) and wild-type CB6F1 mice (60 mice) was shaved (2 × 2 cm area) and the animals received a single topical application (painting) of 0.1 ml DMBA solution (2 mg/ml in acetone). Two weeks later, the animals were divided into 3 groups and the area which was painted with DMBA was shaved and painted with silicon oil alone or $sTiO_2$ suspended in silicon oil 5 times a week until termination of the experiment: Group 1 mice (15 mice of each strain) were painted with 0.2 ml silicone oil; group 2 mice (15 mice of each strain) were painted with 0.2 ml of 50 mg/ml $sTiO_2$ suspended in silicone oil; group 3 mice (15 mice of each strain) were painted with 0.2 ml of 100 mg/ml $sTiO_2$ suspended in silicone oil. Group 4 consisted of 15 mice of each strain painted with 0.2 ml 100 mg/ml $sTiO_2$ suspended in silicon oil 5 times a week without prior DMBA treatment. The rasH2 mice were killed at experimental week 8 and wild-type CB6F1 mice were killed at experimental week 40.

Skin carcinogenesis study of non-coated TiO_2 ($ncTiO_2$) using Hras128 rats

The back skin of 10-week-old male Hras128 rats (50 rats) and wild-type SD rats (36 rats) was shaved (3 × 3 cm area) and the animals received a single topical application (painting) of 0.5 ml DMBA solution (5 mg/ml in acetone)

(Park *et al.*, 2004). Two weeks later, the animals were divided into 3 groups and the area which was painted with DMBA was shaved and painted with Pentalan 408 alone or ncTiO_2 suspended in Pentalan 408 twice a week until termination of the experiment: Group 1 rats (17 Hras128 and 12 SD rats) were painted with 0.5 ml Pentalan 408 alone; group 2 rats (16 Hras128 and 12 SD rats) were painted with 0.5 ml of 100 mg/ml ncTiO_2 suspended in Pentalan 408; group 3 rats (17 Hras128 and 12 SD rats) were painted with 0.5 ml of 200 mg/ml sTiO_2 suspended in Pentalan 408. The Hras128 rats were killed at experimental week 28 and wild-type SD rats were killed at experimental week 40.

Skin carcinogenesis study of non-coated TiO_2 (ncTiO_2) using wild-type CD1 mice

The back skin of 10-week-old female CD1 mice (62 mice) was shaved (2 × 2 cm area) and the animals received a single topical application (painting) of 0.1 ml DMBA solution (2 mg/ml in acetone). Two weeks later, the animals were divided into 4 groups and the area which was painted with DMBA was shaved and painted with Pentalan 408 alone twice a week, ncTiO_2 suspended in Pentalan 408 twice a week, or TPA 4 times a week (positive control) until termination of the experiment: Group 1 mice (16 mice) were painted with 0.2 ml Pentalan 408; group 2 mice (16 mice) were painted with 0.2 ml of 50 mg/ml ncTiO_2 suspended in Pentalan 408; group 3 mice (15 mice) were painted with 0.2 ml of 100 mg/ml ncTiO_2 suspended Pentalan 408; group 4 mice (15 mice) were painted with 0.2 ml TPA solution (200 nmol/ml in acetone). Group 1-3 mice were killed at experimental week 52; group 4 mice were killed at experimental week 40.

Skin penetration study of non-coated TiO_2 (ncTiO_2) in SD rats

Based on our previous study showing lack of TiO_2 penetration through the normal skin (Xu *et al.*, 2011), ncTiO_2 was applied to damaged skin, which is postulated to be more susceptible to particle penetration. The back skin of 10-week-old female SD rats (24 rats) was shaved (3 × 3 cm area) and the epidermis was removed by stripping the epidermis off with a fresh piece of adhesive tape (3M's No. 3760, Scotch Mending Tape, Sumitomo 3M Ltd., Tokyo, Japan): Stripping was done 30 times to completely remove the epidermis. The epidermis-stripped skin was then painted with 0.5 ml of Pentalan or 0.5 ml of 200 mg/ml ncTiO_2 suspended in Pentalan 408 at 4-day-intervals over the course of 3 and a half weeks (7 treatments in 3½ weeks). Localization of ncTiO_2 particles in the epidermis was determined by histological observation. Skin tissue samples were taken at 1, 3, and 7 days after stripping to examine recovery of the epidermis and penetration of ncTiO_2 into the skin.

Skin penetration study of silicone coated TiO_2 (sTiO_2) in the in vitro skin model

To evaluate whether optimally dispersed sTiO_2 particles could penetrate into the epidermis, we applied sTiO_2 particles dispersed in silicone oil to the LabCyte EPI-MODEL kit (Japan Tissue Engineering Co. Ltd., Aichi, Japan), which is constructed of human skin epidermis keratinocytes on a mesh over a receiving chamber. In 12 wells: 4 wells had silicone oil alone applied directly to the human skin epidermis keratinocytes for 48 hr; 4 wells had 100 mg/ml sTiO_2 suspended in silicone oil applied directly to the human skin epidermis keratinocytes for 48 hr; and 4 wells had 200 mg/ml sTiO_2 suspended in silicone oil applied directly to the human skin epidermis keratinocytes for 48 hr. The medium in the receiving chamber was collected for elemental titanium analysis by an inductively coupled plasma/mass spectrometry (ICP-MS) (HP-4500, Hewlett-Packard Co., Houston,TX, USA) as described previously (Xu *et al.*, 2011).

Statistical analysis

Statistical analysis was performed using the Kruskal-Wallis and Bonferroni-Dunn's multiple comparison tests. Statistical significance was analyzed using a two-tailed Student's *t*-test and Bonferroni-Dunn's multiple comparison test. A value of $P < 0.05$ was considered to be significant.

RESULTS

Size distribution of ncTiO_2 and sTiO_2 particles

Transmission electron microscopy (TEM) analysis showed that the shape of sTiO_2 particles was generally round to oval (Fig. 1A), while ncTiO_2 particles were more clubbed shaped (Fig. 1B). The sTiO_2 in silicone oil solutions remained without obvious sedimentation for 3 days after preparation (Fig. 1C). In contrast, the ncTiO_2 in Pentalan 408 solutions contained considerable sedimentation 3 days after preparation (Fig. 1D). The particle size distribution of sTiO_2 and ncTiO_2 solutions is shown in Figs. 1E and 1F. The mean length of sTiO_2 particles suspended in saline and silicone was 0.16 ± 0.07 and 0.28 ± 0.22 µm, respectively (Fig. 1E). The mean length of ncTiO_2 particles suspended in saline and Pentalan 408 was 3.18 ± 0.35 and 4.97 ± 0.50 µm, respectively (Fig. 1F). These results indicate that sTiO_2 in silicone oil remained dispersed for a longer time than ncTiO_2 in Pentalan 408.

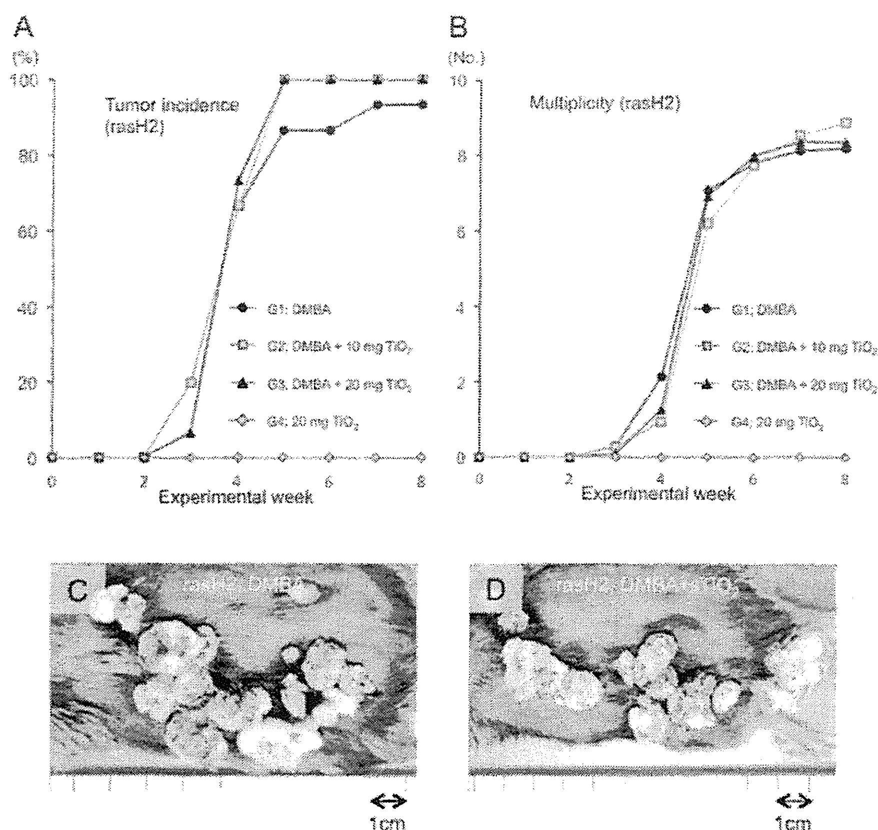

Fig. 2. Effects of $sTiO_2$ in a two-stage carcinogenesis model using rasH2 mice. The incidence of skin tumors in rasH2 mice: DMBA treated group (black circles), DMBA followed by treatment with 10 mg $sTiO_2$ (gray squares) or 20 mg $sTiO_2$ (black triangles). 20 mg $sTiO_2$ treated group (gray diamonds) (A). Multiplicity of skin tumors in rasH2 mice: DMBA treated group (black circles), DMBA followed by treatment with 10 mg $sTiO_2$ (gray squares) or 20 mg $sTiO_2$ (black triangles). 20 mg $sTiO_2$ treated group (gray diamonds) (B). Representative macroscopic appearance of skin tumors initiated with DMBA in rasH2 mice (C). The gross morphology of the tumors did not show obvious differences between the DMBA (C) and DMBA + $sTiO_2$ (D) groups.

Skin carcinogenesis study of $sTiO_2$ in silicone oil using rasH2 mice

Figures 2A and 2B show the time lapse incidence and multiplicity (number/mouse) of macroscopic skin tumors in rasH2 mice. No statistically significant differences were found between $sTiO_2$ treated rasH2 mice (groups 2 and 3: DMBA plus 50 mg/ml and 100 mg/ml $sTiO_2$, respectively) and control rasH2 mice (group 1: DMBA alone) (Figs. 2A and 2B, Table 2-1). Similarly, no statistically significant differences were found between treated and control groups of wild-type CB6F1 mice (Table 2-2). No tumors were induced in group 4 ($sTiO_2$ alone) of either rasH2 (Figs. 2A and 2B, Table 2-1) or wild-type CB6F1 mice (Table 2-2). Skin tumors were histologically SCP and SCC in rasH2 and wild-type CB6F1 mice. Representative macroscopic skin tumors induced by DMBA alone and by DMBA plus $sTiO_2$ are shown in Fig. 2C and 2D, respectively.

Skin carcinogenesis study of $ncTiO_2$ using Hras128 rat

Figures 3A and 3B show the time lapse incidence and multiplicity (number/rat) of macroscopic skin tumors in Hras128 rats. No statistically significant differences were found between $sTiO_2$ treated and control rasH2 rats (Figs. 3A and 3B, Table 3-1). Similarly, no statistically significant differences were found between treated and control groups of wild-type SD rats (Table 3-2).

Microscopically, TiO_2 was not observed within the SCP or SCC tissue (Figs. 4A and 4B). TiO_2 was observed

Table 2-1. Effects of $sTiO_2$ on skin carcinogenesis in rasH2 mice

Group	Treatment	No. of mice	SCP		SCC		SCP + SCC	
			Incidence (%)	Multiplicity	Incidence (%)	Multiplicity	Incidence (%)	Multiplicity
1	DMBA + Silicone	15	14 (93)	7.27 ± 4.74	5 (33)	0.60 ± 0.99	14 (93)	7.87 ± 5.17
2	DMBA + 10 mg TiO_2	15	15 (100)	8.13 ± 3.66	9 (60)	1.00 ± 1.00	15 (100)	9.13 ± 3.76
3	DMBA + 20 mg TiO_2	15	15 (100)	6.80 ± 3.88	8 (53)	0.73 ± 0.80	15 (100)	7.53 ± 3.31
4	20 mg TiO_2	15	0	0	0	0	0	0

SCP, squamous cell papilloma; SCC, squamous cell carcinoma.
Multiplicity: number of tumors per mouse.

Table 2-2. Effects of $sTiO_2$ on skin carcinogenesis in wild-type CB6F1 mice

Group	Treatment	No. of mice	SCP		SCC		SCP + SCC	
			Incidence (%)	Multiplicity	Incidence (%)	Multiplicity	Incidence (%)	Multiplicity
1	DMBA + Silicone	15	1 (7)	0.07 ± 0.26	1 (7)	0.07 ± 0.26	2 (13)	0.13 ± 0.35
2	DMBA + 10 mg TiO_2	15	2 (13)	0.13 ± 0.35	0	0	2 (13)	0.13 ± 0.35
3	DMBA + 20 mg TiO_2	15	2 (13)	0.20 ± 0.56	0	0	2 (13)	0.20 ± 0.56
4	20 mg TiO_2	15	0	0	0	0	0	0

SCP, squamous cell papilloma; SCC, squamous cell carcinoma.
Multiplicity: number of tumors per mouse.

on the surface and in the upper SC tissue and upper part of the hair follicles, but not in the underlying epidermis, dermis or subcutaneous tissues (Figs. 4C and 4D).

Skin carcinogenesis study of $ncTiO_2$ using wild-type CD1 mice

No statistically significant differences in tumor incidence or multiplicity was observed between treated and control groups of CD1 mice (Table 4). TPA treatment after DMBA significantly increased the incidence and multiplicity of SCP ($P < 0.001$).

Skin penetration study of $ncTiO_2$ in SD rats

Figures 5 A-D shows skin tissue samples collected before (Fig. 5A) and 1, 3 and 7 days after (Figs. 5B-D) removing the epidermis by tape-stripping. On day 1 the epidermis was completely removed (Fig. 5B), and a mass of fibrin exudate and underlying granulation tissue rich with neutrophils was the main feature of the skin surface. On day 3, regenerated epidermis already covered the granulation tissue (Fig. 5C). On day 7, the surface of the skin was fully covered by regenerated keratinocytes showing cornification and had an almost normal appearance (Fig. 5D).

The shaved back skin of SD-rats was painted with 100 mg TiO_2 suspended in Pentalan 408. Fig. 5E shows the presence of $ncTiO_2$ particles in the SC layer of the skin of these animals. Extensive histological observation failed to detect TiO_2 within the epidermis or dermis. In another series of experiments, the epidermis was removed from the back skin of SD rats by tape-stripping and the freshly stripped skin was painted with 100 mg $ncTiO_2$ suspended in Pentalan 408. This was repeated every 4 days for 3½ weeks (7 times total). Figure 5F shows the presence of TiO_2 on the surface of the regenerating epidermis 1 day after the last stripping/painting procedure. Extensive

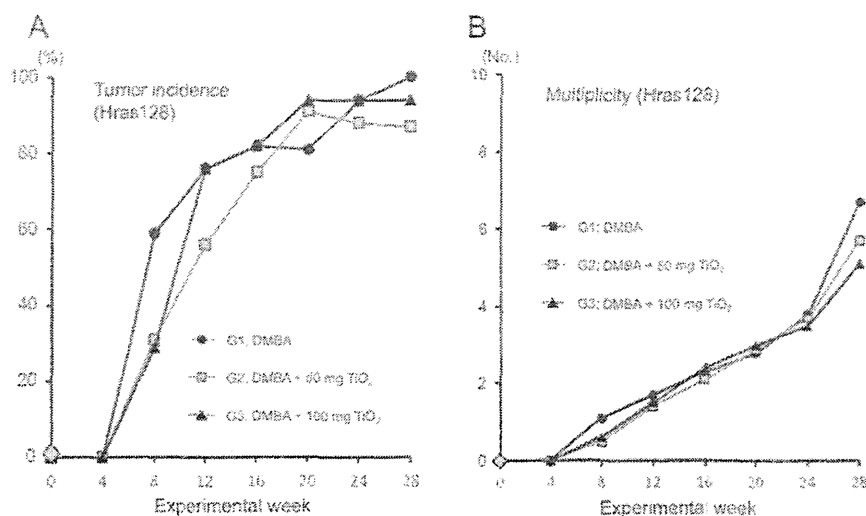

Fig. 3. Effects of ncTiO_2 in a two-stage carcinogenesis model using Hras128 rats. The incidence of skin tumors in Hras128 rats: DMBA treated group (black circles), DMBA followed by treatment with 50 mg ncTiO_2 (gray squares) or 100 mg ncTiO_2 (black triangles) (A). Multiplicity of skin tumors in rasH2 mice: DMBA treated group (black circles), DMBA followed by treatment with 100 mg ncTiO_2 (gray squares) or 200 mg ncTiO_2 (black triangles) (B).

Table 3-1. Effects of ncTiO_2 on skin carcinogenesis in Hras128 rats

Group	Treatment	No. of rats	SCP		SCC		SCP + SCC	
			Incidence (%)	Multiplicity	Incidence (%)	Multiplicity	Incidence (%)	Multiplicity
1	DMBA + Pentalan 408	17	16 (94)	9.65 ± 7.05	0	0	16 (94)	9.65 ± 7.05
2	DMBA + 50 mg TiO_2	16	14 (88)	6.81 ± 6.21	2 (13)	0.19 ± 0.54	14 (88)	7.00 ± 6.52
3	DMBA + 100 mg TiO_2	17	16 (94)	7.59 ± 3.86	2 (12)	0.12 ± 0.331	16 (94)	7.71 ± 3.93

SCP, squamous cell papilloma; SCC, squamous cell carcinoma.
Multiplicity: number of tumors per rat.

Table 3-2. Effects of ncTiO_2 on skin carcinogenesis in wild-type SD rats

Group	Treatment	No. of rats	SCP		SCC		SCP + SCC	
			Incidence (%)	Multiplicity	Incidence (%)	Multiplicity	Incidence (%)	Multiplicity
1	DMBA + Pentalan 408	12	3 (25)	0.25 ± 0.45	0	0	3 (25)	0.25 ± 0.45
2	DMBA + 50 mg TiO_2	12	2 (17)	0.17 ± 0.39	2 (17)	0.17 ± 0.39	4 (33)	0.33 ± 0.49
3	DMBA + 100 mg TiO_2	12	1 (8)	0.08 ± 0.29	0	0	1 (8)	0.08 ± 0.29

SCP, squamous cell papilloma; SCC, squamous cell carcinoma.
Multiplicity: number of tumors per rat.

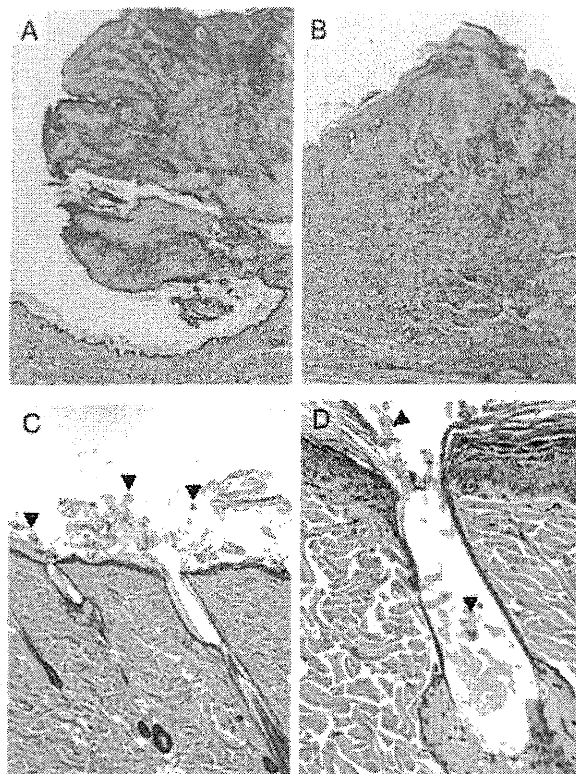

Fig. 4. Effects of ncTiO_2 in a two-stage carcinogenesis model. Representative histological features of SCP (A) and SCC (B) on the back skin of a Hras128 rat. TiO_2 aggregates were seen on the surface of stratum corneum (C, arrowheads), but not in the underlying epidermis, dermis or subcutaneous tissue. Some aggregates were found in the upper layer of stratum corneum (arrowheads) and in the lumen of the hair follicle (D) but not in the dermis.

histological observation failed to detect any TiO_2 particles in the underlying epidermis or dermis, indicating that ncTiO_2 failed to penetrate into the stripped skin. In addition, no inflammatory lesions were found in the epidermis or subcutaneous tissue of these animals. These results indicate that TiO_2 particles do not penetrate into the epidermis of either normal or damaged skin.

In vitro skin penetration study

The amount of elemental titanium in the receiving chamber did not show any significant increase over the vehicle group (Table 5). All the observed values are equivalent to background. The results indicate that sTiO_2 particles did not penetrate the human epidermis in this model.

DISCUSSION

TiO_2 particles, including both nano and larger sized particles, are known to be carcinogenic to the rat lung (Baan *et al.*, 2006). We have shown that alveolar macrophages play an important role in promotion of lung carcinogenesis when TiO_2 particles are inhaled into the lung (Xu *et al.*, 2011). Because of this, TiO_2 particles, especially nano-sized particles, are deemed to have the potential to induce skin tumors after long-term topical application should the particles penetrate into the epidermis and subcutaneous tissue and interact with macrophages. The current study is the first systematic study of the skin promotion/carcinogenesis effects of TiO_2 in a two-stage chemical carcinogenesis animal model. We found that even the smallest available sized TiO_2 (sTiO_2) did not exhibit promoting effects on the highly sensitive rasH2 mouse skin carcinogenesis model. Furthermore, we observed that TiO_2 without coating (ncTiO_2) did not cause skin tumor promotion in the skin carcinogenesis-sensitive Hras128 rat model or in the CD1 mouse. These results are in agreement with another recent study reporting the lack of carcinogenicity of topically applied TiO_2 (Furukawa *et al.*, 2011).

We also found that topically applied ncTiO_2 did not penetrate normal rat skin or skin which had the epidermis completely removed, nor did sTiO_2 penetrate the *in vitro* human epidermis model. Thus, the lack of skin promotion/carcinogenesis effects is probably due to lack of penetration of the particles through the epidermis to the dermis where cytogenetic cells of skin carcinogenesis reside. In another study, we found no promoting effect of TiO_2 particles in a UVB-initiated long-term (52 weeks) skin carcinogenesis study and no penetration of TiO_2 particles through the epidermis (Xu *et al.*, 2011).

Results showing lack of TiO_2 penetration through the epidermis are in accordance with other reports. Numerous *in vitro* and *in vivo* studies using murine, porcine, or human skin have shown that nano-sized TiO_2 does not penetrate the skin (reviewed in Nohynek *et al.* (2008)). In addition to these studies, Gottbrath *et al.* (2003) report that after topical application of TiO_2 to the underside of the forearm of a human volunteer, ultrafine TiO_2 did not penetrate beyond the SC.

Contrary to these findings, there is a single report by Wu *et al.* (2009) that TiO_2 particles (4 and 60 nm) did penetrate into the deep layers of the epidermis after topical application to the pig ear for 30 days (Wu *et al.*, 2009). They also reported that nano-size TiO_2 particles could penetrate the skin of hairless mice after 60 days dermal exposure, although they did not examine promotion/car-

Table 4. Effects of $ncTiO_2$ on skin carcinogenesis in wild-type CD1 mice

Group	Treatment	No. of mice	SCP		SCC		SCP + SCC	
			Incidence (%)	Multiplicity	Incidence (%)	Multiplicity	Incidence (%)	Multiplicity
1	DMBA + Pentalan 408	16	3 (19)	0.25 ± 1.30	0	0	3 (19)	0.25 ± 0.58
2	DMBA + 10 mg TiO_2	16	1 (6)	0.06 ± 0.25	0	0	1 (6)	0.06 ± 0.25
3	DMBA + 20 mg TiO_2	15	2 (13)	0.13 ± 0.35	0	0	2 (13)	0.13 ± 0.35
4	DMBA + TPA	15	13 (87)*	2.00 ± 1.41*	2 (13)	0.13 ± 0.35	13 (87)*	2.00 ± 1.41*

* Significantly different from group 1 (control) by Student's t-test ($p < 0.001$).
SCP, squamous cell papilloma; SCC, squamous cell carcinoma.
Multiplicity: number of tumors per mouse.

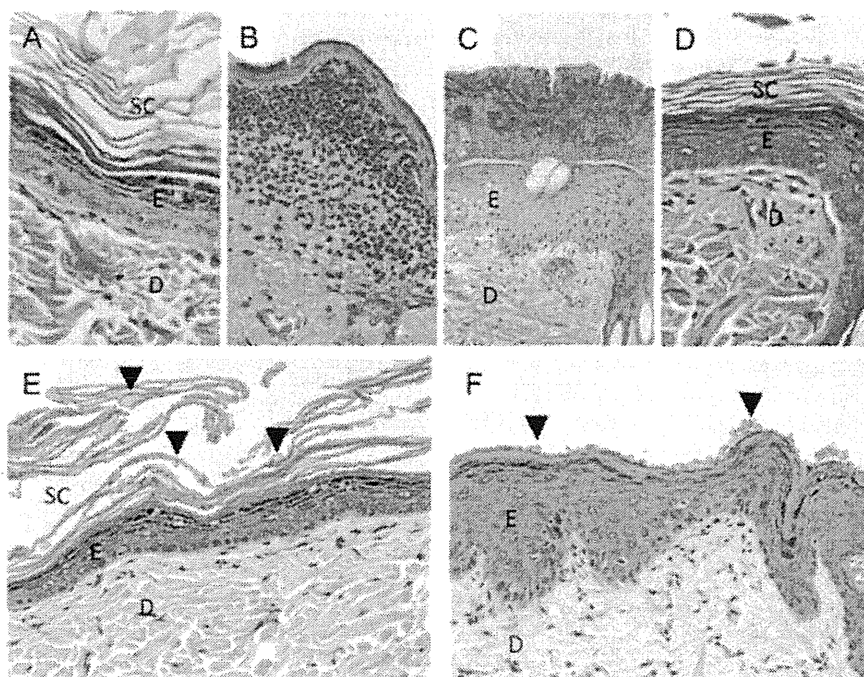

Fig. 5. Histological features of the skin after striping away the epidermis and study of $ncTiO_2$ penetration into normal and stripped skin using wild-type Sprague-Dawley rats. The back skin of SD rats was shaved and the epidermis was left intact or stripped away using tape. Stratum corneum (SC), epidermis (E), and dermis (D) are intact in the shaved, not stripped group (A). In the tape-stripped group, one day after stripping away the epidermis, the stripping site is covered with fibrin exudates with rich neutrophilic infiltration (B). Three days after stripping, regenerated epidermis (E) is present underneath the exudate (C). Seven days after stripping, the regenerated epidermis (E) is composed of mature keratinocytes and a stratum corneum (SC) (D). The back skin of SD rats was shaved (but the epidermis was not stripped away) and painted with $ncTiO_2$ suspended in Pentalan 408. $ncTiO_2$ aggregates are present in the stratum corneum (SC) (arrowheads, brown material) of the skin of these animals (E); particles were not detected within the underlying skin tissue. Freshly stripped skin was painted with $ncTiO_2$ suspended in Pentalan 408, and this process was repeated every 4 days over the course of $3^1/_2$ weeks (7 times total). $ncTiO_2$ particles (arrowheads, brown material) are present on the surface of the skin one day after the last stripping/painting procedure (F); particles were not detected in the underlying tissue.

Table 5. *In vitro* penetration of $sTiO_2$ particles

Treatment	Amount of elemental titanium in the receiving chamber (μg/ml)
Silicone oil	0.11 ± 0.01
100 mg/ml $sTiO_2$	0.14 ± 0.01
200 mg/ml $sTiO_2$	0.12 ± 0.01
None	0.11 ± 0.03

cinogenesis effects of the particles (Wu *et al.*, 2009). Differences in experimental systems (i.e., animal strain used in the experiment, exposure period, particle suspension, mean primary/actual length of the particle, and TiO_2 manufacturer) may possibly explain the discrepancies reported on TiO_2 particle penetration.

Two studies have reported finding TiO_2 particles in hair follicles (Bennat and Muller-Goymann, 2000; Lekki *et al.*, 2007). These studies taken together with Wu *et al.* (2009) suggest the possibility that the hair follicle may be a route of skin penetration by TiO_2 particles. However, we found that TiO_2 particles remained primarily in the SC and the upper lumen of hair follicles. Bennat and Muller-Goymann (2000) and Lekki *et al.* (2007) also report that while topically applied TiO_2 was found in hair follicules, it did not penetrate into the underlying tissue or sebaceous glands.

Penetration of TiO_2 into underlying dermal tissues even after removing the entire epidermis by tape-stripping did not occur. Rather, aggregates of TiO_2 particles were found on the exterior of the SC exhibiting regeneration and no particles were found in the underlying tissues. The freshly manufactured TiO_2 particles used in the present study primarily measured 20-35 nm in their longer diameter. These particles are lyophobic and easily form micro-sized aggregates (160-5,000 nm in length), and these larger particles are unable to penetrate through the epidermis.

In summary, nano-sized TiO_2 particles, even silicone coated TiO_2 suspended in silicone oil which provides optimal dispersion of the particles, did not penetrate the epidermis of rat or human skin models and did not exhibit promoting effects in rat or mouse two-stage skin carcinogenesis models. Therefore, topical application of TiO_2 was not carcinogenic, and this lack of carcinogenicity is likely due to the lack of penetration through the epidermis. Our studies taken together with other reports lead us to conclude that topical application of TiO_2 to human skin is very unlikely to pose a potential risk to human health.

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