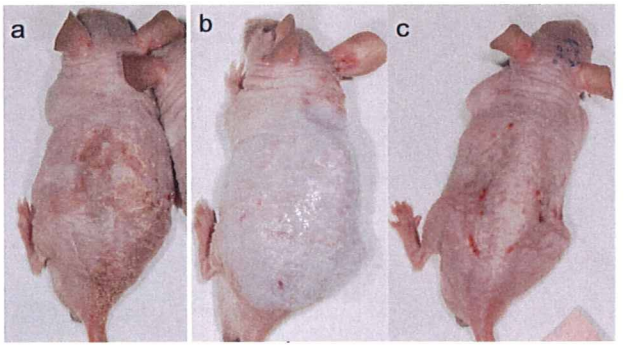
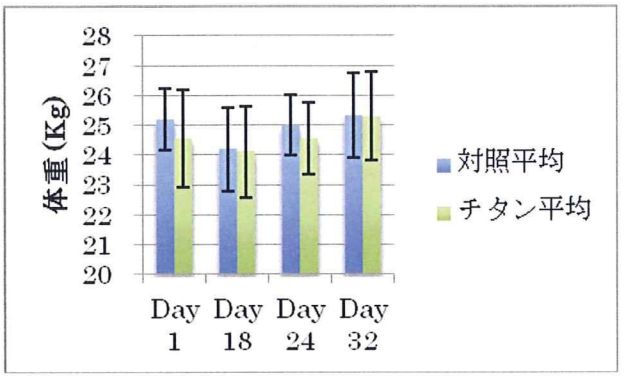


図 10.皮膚炎マウス体重変化

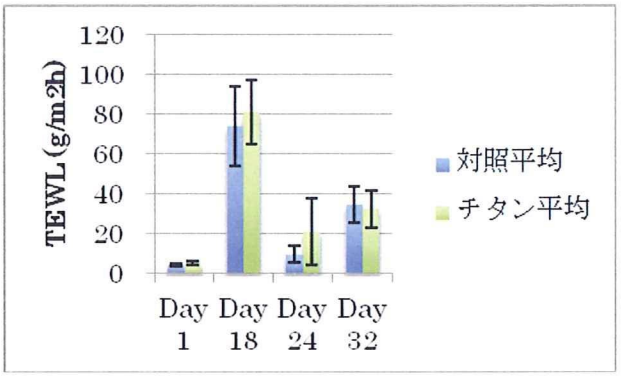


2）-1.皮膚生理指標

TEWL

皮膚炎惹起後、DNFB で更に皮膚炎が重度になるのを防ぐため、二酸化チタン塗布中は DNFB を Day 24 に 1 回だけ塗布して軽度から中等度の皮膚炎を維持した。二酸化チタン外用投与群の TEWL は、DNFB 前（Day 1）、5.3±0.7g/m^{2}h (対照 4.3±0.7)、二酸化チタン投与前 （Day 18）、81.3±16.2（対照 74.2±20.2）、二酸化チタン投与中（Day 24）、21.2±16.7（対照 9.8±4.2）、二酸化チタン投与終了時（Day 32）、32.5±9.3（対照 34.7±9.2）であり、DNFB で惹起された皮膚炎により皮膚バリア機能指標である TEWL 値は上昇したものの、二酸化チタン投与群と溶媒対照群とで有意差を認めなかった（図 11）。

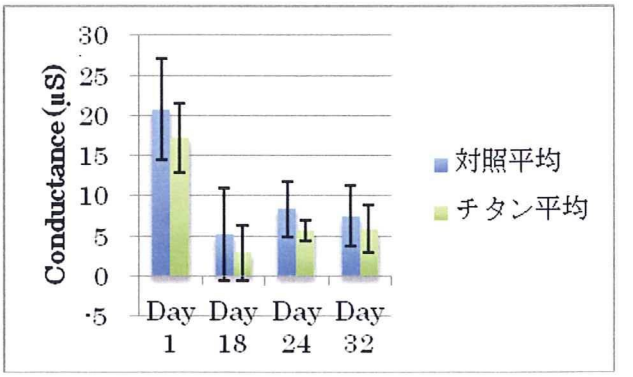
図 11. 皮膚炎マウスに二酸化チタン外用投与後の TEWL 値（皮膚バリア機能指標）の推移



高周波コンダクタンス

二酸化チタン外用投与群の高周波コンダクタンスは、DNFB 前（Day 1）、17.2±4.4 μS (対照 20.8±6.3)、二酸化チタン投与前 （Day 18）、2.9±3.4 (対照 5.2±5.7)、二酸化チタン投与中 (Day 24)、5.7±1.2（対照 8.4±3.4）、二酸化チタン投与終了時（Day 32）、5.9±2.9（対照 7.5±3.8）であり、DNFB で惹起された皮膚炎により角層水分量指標である高周波コンダクタンス値は低下したものの、その後二酸化チタン投与群と溶媒対照群とで有意差を認めなかった（図 12）。

図 12. 皮膚炎マウスに二酸化チタン外用投与後の高周波コンダクタンス値（角層水分量指標）の推移

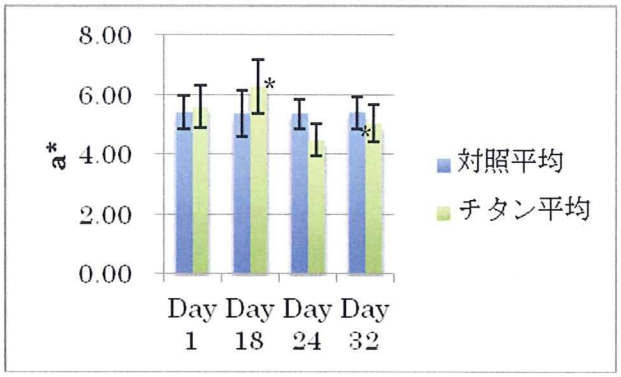


紅斑（赤み）

二酸化チタン外用投与群の a*は、DNFB 前（Day 1）、5.60±0.72 (対照 5.41±0.57)、二酸化チタン投与前（Day 18）、6.27±0.9 (対照 5.37±0.79)、二酸化チタン投与中（Day 24）、4.48±0.55（対照 5.35±0.48）、二酸化チタン投与終了時（Day 32）、5.04±0.64（対照 5.40±0.54）であり、Day 18

で二酸化チタン投与群が有意に高値、Day 24 で対照群が有意に高値であったが、Day 32 では両群で有意な差を認めなかった（図 13）。

図 13. 皮膚炎マウスに二酸化チタン外用投与後の a*値（皮膚色の赤み）の推移



2)-2.血中好酸球%、IgE

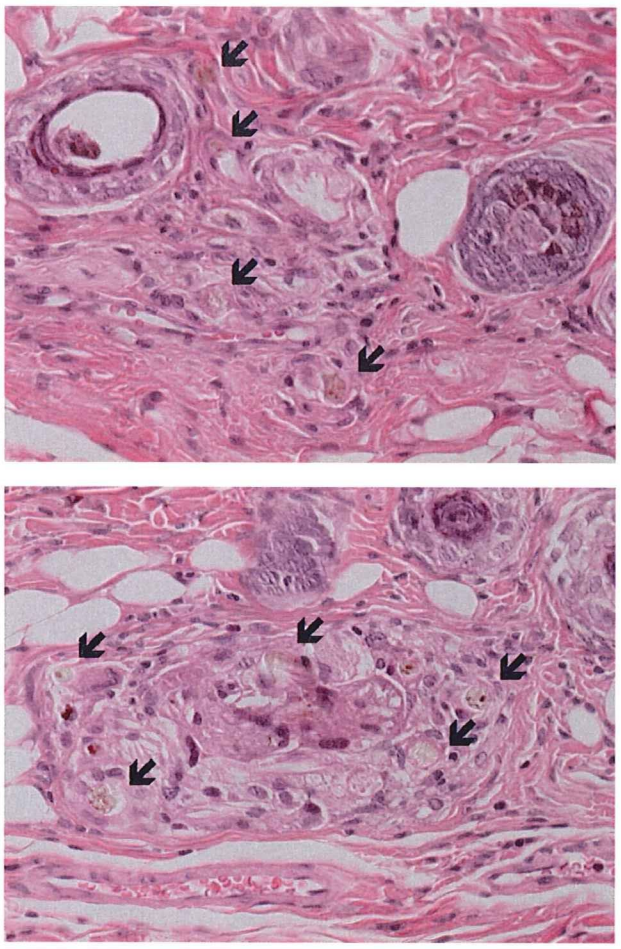
好酸球%は、二酸化チタン群 2%に対し、対照 3%と両群とも上昇していなかった。採血量が少なかたマウスは IgE 測定が出来なかった。二酸化値チタン群 105.2ng/ml（n=7）、対照群 108.4 ng/ml (n=4)と両群で有意差はなかった。

2）-3.病理組織

皮膚

二酸化チタン外用投与皮膚で、真皮内にチタン粒子と思われる沈着物とその周囲に肉芽腫の形成が認められた（図 14）。表皮の海綿状態、炎症細胞浸潤、真皮の炎症細胞浸潤は、対照も多数みられるので、CD45 の免疫染色を行い検討中である。

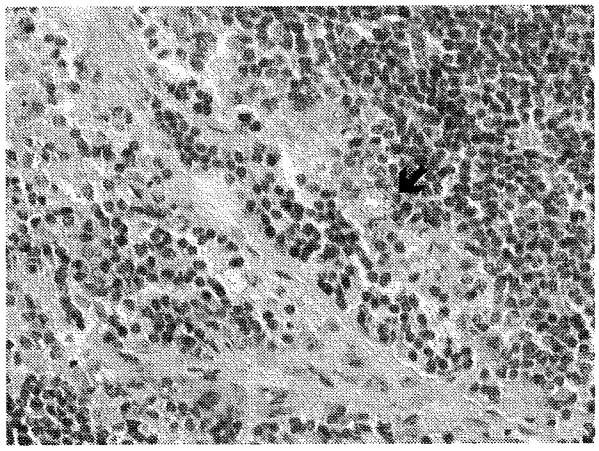
図 14. DNFB 惹起した皮膚炎のある背部皮膚に二酸化チタンを連続外用投与したマウス皮膚病理組織。茶色の沈着物（矢印）を取り囲む肉芽腫の形成を認める。



リンパ節

途中で死亡した 1 匹（対照群）を除き、19 匹から Day 32 にリンパ節を摘出した。二酸化チタン群 10 匹中 1 匹のリンパ節に、二酸化チタン粒子と思われる沈着物を認めた（図 15）。

図 15.　DNFB 惹起した皮膚炎のある背部皮膚に二酸化チタン外用投与後、摘出したリンパ節。二酸化チタンと思われる沈着物を認めた（矢印）。



D．考察

シリコンコーティング一次粒子径 20nm の二酸化チタンを溶媒シリコン D5 に分散剤を用いて分散させ 100mg/ml の濃度に調整したものを試験試料として用いた。これは一般にサンスクリーン剤に配合されている二酸化チタン濃度約 10%としてほぼ相当すると考えられる。

角層をテープストリッピングで剥離して作成した損傷皮膚に二酸化チタンを単回外用投与し、１時間後、３時間後の病理組織ならびに電顕での観察では、二酸化チタンが生きた表皮や真皮に吸収されるという証拠は得られず、投与皮膚局所で生体皮膚計測工学的手法を用いた皮膚生理機能での評価でも、溶媒対照と比較して、より皮膚に障害を与えたというデータは得られなかった。

DNFB を塗布して慢性的に皮膚炎を惹起させたマウス皮膚に、連続して二酸化チタンを外用投与した場合、真皮、さらに、所属リンパ節にチタン粒子と考えられる沈着物を認めた。皮膚では、沈着物を取り囲む肉芽腫の形成が認められた。投与皮膚局所で生体皮膚計測工学的手法を用いた皮膚生理機能での評価では、この皮膚炎モデルにおいても、溶媒対照と比較して、より皮膚に障害を与えたというデータは得られなかった。一般にアトピー性皮膚炎で高値を示す血中好酸球%や血中 IgE は、二酸化チタン投与マウスと対照間で有意な差を認めず、皮疹部への二酸化チタンの外用投与により全身的にアトピー性皮膚炎を増悪させる証拠は得られなかった。しかし、慢性的に皮膚炎があるような障害皮膚に長期に二酸化チタンを外用投与した場合、皮膚から吸収され、皮膚では真皮に沈着し肉芽腫を形成すること、さらにリンパ流に入り全身に移行する可能性があることが示された。これがナノ粒子であるためか、ナノ粒子でなくても二酸化チタンでは同様なことが起こるのか検討が必要と考えられる。

E．結論

アトピー性皮膚炎など慢性的に皮膚障害がある皮膚に、長期にわたり二酸化チタンを外用投与することにより、皮膚から二酸化チタンが吸収される可能性が示された。約２週間という期間で観察した限り、皮膚局所の皮膚炎の増悪や全身的な皮膚炎の増悪はみられなかったが、吸収された二酸化チタンが何らかの生体学的に有害な作用をもたらす可能性は否定できず、今後さらに検討が必要である。

F．健康危機情報

皮膚炎の病変部にナノ粒子二酸化チタンを外用投与すると、経皮吸収され、所属リンパ節に運ばれ、全身に移行する可能性がある。皮膚局所では肉芽腫の形成がみられる。

F．研究発表

1．論文発表

なし

2．学会発表

なし

G．知的財産権の出願・登録状況（予定を含む）

1．特許取得

なし

2．実用新案登録

なし

3．その他

平成21年度　厚生労働科学研究費補助金（化学物質リスク研究事業）
分担研究報告書

研究課題名：ナノマテリアルの経皮毒性に関する評価手法の開発に関する研究

分担研究課題名：*In vivo* 経皮吸収慢性評価のパラメータの検索

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五十嵐良明　国立医薬品食品衛生研究所・環境衛生化学部　室長
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研究要旨

今までの実験に用いたルチル型・非コーテイング・ペンタラン分散のナノ粒子二酸化チタン（nTD、凝集塊の平均粒径3.18μm）は健常皮膚に塗布した場合には、発がんプロモーション作用を示さ無いことが分かった。今回はnTDの高度障害皮膚の透過性と、より分散性の高いシリコンコーティング．シリコンオイル分散nTD粒子（SnTD凝集塊直径164nm）の皮膚発がん透過性とプロモーション作用を検討した。nTDはラットにおいて角質をほぼ完全に除去した高度障害背部皮膚でも透過しなかった。SnTDは皮膚発がん感受性の高いrasH2マウスに今までと同じ発がん二段階モデルにてプロモーション作用を検討したがプロモーション作用を示さなかった。また、野生型マウスを用いた検討でも同様結果であった。これらマウスの実験においても皮膚透過性は無かった。これらの結果から、nTDはラット皮膚を透過しない、さらに分散性の高い二酸化チタニウムは、マウス正常皮膚に対し皮膚を通過せず、発がん性も示さないことが示された。

A．研究目的

ナノ材料は多分野における全く新しい素材として世界中で開発が進められている。このうち、ナノ粒子二酸化チタニウム（nTD）は既に上市され、シリコンコートした二酸化チタニウム粒子が化粧品として皮膚に塗布されることが多い。本年度は、分散性の高いシリコンnTDの皮膚発がん性に対する影響を検討する目的で、以下の検討をおこなった。

実験１）いままでのラット研究において、ルチ

ル型・非コーテイング・ペンタラン分散のナノ粒子二酸化チタン（nTD）は健常皮膚を通過しないことが分かったので、障害皮膚の通過性を検討。

実験２）より分散性の高いシリコンコーティング．シリコンオイル分散 nTD 粒子（SnTD）の粒子の大きさの分布の確認。

実験３）皮膚発がん感受性の高い rasH2 マウスを用いた SnTD の皮膚発がん二段階モデルによる発がんプロモーション作用の検討。

B．研究方法

実験１）nTD の障害皮膚の透過性の検討：8 週齢 SD 系雄ラットの背部皮膚（3 x 3 cm^2）において粘着テープによって 30 回ストリッピングを行って角質除去を行った直後に nTD を 0.5ml 塗布して 7 日まで経時的に屠殺剖検し通過性を観察した。さらに毛嚢の無い状態での透過性確認のためにヒトケラチノサイトを多層培養した人工皮膚組織の表面に 250 および 500ppm の nTD、100mg を 0.5ml 塗布して 48 時間後に隔膜下のレセプター層への移行を検討した。

実験２）SnTD の粒子の大きさの分布：シリコン SnTD（一次粒径 35nm・ルチル型・シリコンコーティング・シリコン分散液、および従来の nTD（直径 20nm・ルチル型・無コーティング・ペンタラン懸濁液）を作成し、島津レーザー解析式粒度分布測定装置 SALD-7100 を用いて、粒度分布を相対粒子量（%）として測定した。

実験３）皮膚発がん感受性の高い rasH2 マウスを用いた皮膚発がんに段階モデルによる発がんプロモーション作用の検討

皮膚発がん高感受性の雄 CByB6F1-Tg(HRAS)2Jic マウス(rasH2 マウス)および同腹野生型マウスの背部を 2x2cm の大きさに剃毛し皮膚に発がん物質 7,12-dimethylbenz [a] anthracene (DMBA, 300ug/0.1m アセトン)を滴下した。50mg または 100mg の SnTD をシリコン 0.2ml に懸濁し、週 5 回塗布した。rasH2 マウスは 8 週、野生型マウスは 40 週にて屠殺剖検した。

（倫理面への配慮）

実験は動物保護および倫理指針を遵守し、名古屋市立大学動物実験倫理委員会の審査を経て実験を実施した。

C．研究結果

実験１）浅いテープストリッピングでは nTD は角質内と毛嚢腔内に少量見られた。30 回のストリッピングによるほぼ全層角質剥離の場合でも、真皮、皮下組織への移行はなかった。浅い剥離では残存上皮が、全層剥離の場合には残存毛嚢上皮が浸出物または肉芽とその下の真皮との境界部に移行（migrate）しつつ急速に増殖してわずか３日で修復し、nTD を痂皮ごと押し上げて落屑排除してしまうために、真皮、皮下組織には全く見られなかった。毛嚢組織の全く無い人工皮膚において通過性は認めなかった。

実験２）SnTD の平均粒子径は 0.16±0.074 μm で、0.13−0.17 μm の粒子径が全体の 80%を占め、分布の中間値は 0.16 μm であった。また、nTD の平均粒子径は 3.18±0.35 μm で、1.3−5.1 μm の粒子径が全体の 80%を占め、分布の中間値は 3.57 μm であった。

実験３）皮膚発がん高感受性 rasH2 マウスを用いた SnTD の皮膚発がんプロモーション作用の検討：rasH2 マウスの塗布部には、DMBA 投与群のいずれにも 93-100%の発生頻度で皮膚腫瘍性病変が観察され、病理組織学的に検索すると扁平上皮乳頭腫および扁平上皮がんであった。これらの皮膚腫瘍性病変（扁平上皮乳頭腫＋扁平上皮がん）の平均発生個数は、溶媒対照群

8.0±5.4 に対し、シリコン SnTD 50mg 群および 100mg 群ではそれぞれ、9.0±3.7 および 7.5±3.7 であり、発生個数に有意な差は見られなかった。さらに、rasH2 の野生型マウスである C57Black を用いた検索では、肉眼的に皮膚腫瘍性病変が観察されたマウスはいずれの群でも数例で、その発生頻度は、溶媒対照群で 6.7%、SnTD 50mg 群および 100mg 群のいずれも 14.3%であった。

D．考察

ラットにおいて nTD は角質全層剥離した障害皮膚では、残存した表皮または毛嚢上皮が迅速に再生増殖して３日で修復してしまい、nTD を痂皮ごと押し上げて排除してしまうという、異物侵入に対する防御機構のために表皮を浸透通過することはなかった。過去およびこれらの実験において nTD は毛嚢に少量侵入することが観察されたので、毛嚢の無い人工皮膚で透過性を見たところ２４時間後（２０年度）と今回４８時間の実験（昨年度は２４時間観察）で透過しないことが確認された。

次に、皮膚発がん感受性の高い rasH2 マウスによる発がん二段階モデルを用いた、皮膚発がんプロモーション作用の検討では、SnTD はプロモーション作用を示さなかった。また、野生型マウスを用いた検討でもプロモーション作用は見られなかった。このことは、発がん性について、分散性の高い粒子径の遥かに小さい SnTD を用いてもプロモーション作用を示さないことが分かった。

E．結論

H19年度より用いてきたnTDはラットにおいて高度の障害状態でも皮膚組織を透過することは無かった。

シリコンコーティングした二酸化チタニウムは、シリコン分散剤中では分散性が高く小さな粒子径のまま保たれていた。しかし、この二酸化チタニウムを皮膚に塗布しても、皮膚発がん性を示さないことが示された。従って、これらの結果から分散性の高い二酸化チタニウムは、正常皮膚に対し皮膚発がん性を示さないことが示唆された。

以上から、皮膚通過性実験においてナノマテリアルの皮膚通過性の試験法として、マウス、ラットの皮膚は有用な情報をもたらす。皮膚発がん性については、発がん物質を塗布後に検体を塗布する二段階モデルが有用と考えられた。

F．健康危機情報

特記すべきことなし

G. 研究発表

1．論文発表

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H. 知的財産権の出願・登録状況（予定を含む）

1. 特許取得（出願中）

特許名称：ナノ粒子の吸入暴露による発がんのリスクマーカーおよびその用途
発明者：津田洋幸、二口　充、徐結苟
出願人：公立大学法人名古屋市立大学、
公開番号：特願2009-071951
出願日：平成21年3月24日

2. 特許名称：PCT出願「ナノ粒子の吸入暴露による発がんのリスクマーカーおよびその用途」
発明者：津田洋幸、二口　充、徐結苟
出願人：津田洋幸、
公開番号：公開準備中

出願日：平成21年9月25日

2. 実用新案登録

該当なし

3. その他

該当なし

Ⅲ．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

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
Mika Senzui, Toshiaki Tamura, Keiko Miura, Yoshiaki Ikarashi, Yoshiteru Watanabe, Makiko Fujii.	Study on penetration of titanium dioxide nanoparticles into intact and damaged skin *in vitro*	J. Toxicol. Sci.	35	107-113	2010
Iigo M, Alexander DB, Long N, Xu J, Fukamachi K, Futakuchi M, Takase M, Tsuda H.	Anticarcinogenesis pathways activated by bovine lactoferrin in the murine small intestine.	Biochimie	91	86-101	2009
Tsuda H, Futakuchi M, Fukamachi K, Shirai T, Imaida K, Fukushima S, Tatematsu M, Furukawa F, Tamano S, Ito N.	A medium-term, rapid rat bioassay model for the detection of carcinogenic the potential of chemicals.	Toxicol Pathol	38	182-187	2010

Xu J, Futakuchi M, Iigo M, Fukamachi K, Alexander DB, Shimizu H, Sakai Y, Tamano S, Furukawa F, Uchino T, Tokunaga H, Nishimura T, Hirose A, Kanno J, Tsuda H.	Involvement of macrophage inflammation protein 1α (MIP1α) in promotion of rat lung and mammary carcinogenic activity of nano-scale titanium dioxide particles administered by intra-pulmonary spraying.	Carcinogenesis,	31	927-935	2010
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IV. 研究成果の刊行物・別冊

The Journal of Toxicological Sciences (J. Toxicol. Sci.)
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Letter

Study on penetration of titanium dioxide (TiO_2) nanoparticles into intact and damaged skin *in vitro*

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ABSTRACT — It is important for toxicological assessment of nanoparticles to determine the penetration of nanoparticle in skin qualitatively and quantitatively. Skin penetration of four different types of rutile titanium dioxide (TiO_2) (T-35, 35 nm, non-coating; TC-35, 35 nm, with almina/silica/silicon coating; T-disp, 10 x 100 nm, mixture of almina coated and silicon coated particles, dispersed in cyclopentasiloxan; T-250, 250 nm, non-coating) was determined with *in vitro* intact, stripped, and hair-removed skin of Yucatan micropigs to study the effect of dispersion and skin conditions. The TiO_2 was suspended in a volatile silicone fluid used for cosmetics, cyclopentasiloxane, at a concentration of 10%. The suspension was applied at a dose 2 μl/cm^2 for 24 hr, followed by cyanoacrylate stripping. The Ti concentration in skin was determined by ICP-MS. T-35 and T-250 easily aggregated in suspension with a mean diameter greater than 1 μm. TC-35 and T-disp showed good dispersion properties with a mean diameter in suspension of approximately 100 nm. No penetration was observed regardless of TiO_2 type in intact and stripped skin. The concentration of Ti in skin was significantly higher when TC-35 was applied on hair-removed skin. SEM-EDS observation showed that Ti penetrated into vacant hair follicles (greater than 1 mm below the skin surface), however, it did not penetrate into dermis or viable epidermis.

Key words: Nanoparticle, Skin penetration, Hair-removed skin, Stripped skin, Titanium oxide

INTRODUCTION

Titanium dioxide (TiO_2) has been used for cosmetics and is considered to be safe for topical use. Recently, TiO_2 nanoparticles (particle size < 100 nm) are used commonly in cosmetics because of their high transparency in visible wavelengths but high attenuation for UV wavelengths (Popov *et al.*, 2005). However, the safety of different conventional size particles is a concern. Safety concerns are based on not only its toxicity characteristics, but also on the potential increase in amount of absorption. In theoretical, materials with an appropriate octanol/water partition coefficient and low molecular weight (< *ca.* 500) penetrate skin through the stratum corneum (SC); therefore, penetration of inorganic particles into intact skin is not possible (Roberts *et al.*, 2002). Some studies indicate that TiO_2 and other inorganic particles, even on a nano-grade scale, do not penetrate skin (Schulz *et al.*, 2002; Pinheiro *et al.*, 2007; Nohynek *et al.*, 2008). However, some nanoparticles can penetrate viable skin (Ryman-Rasmussen *et al.*, 2006; Menzel *et al.*, 2004). Inorganic particles are often lyophobic in both water and oil, dispersed particles easily aggregate to form large (micro-grade) particles. A few studies investigating both dispersibility and skin penetration have been reported (Bennat and Müller-Goymann, 2000). The present study focused on skin penetration of TiO_2 *in vitro* with different dispersibility of TiO_2 and skin condition.

MATERIALS AND METHODS

Materials

All types of TiO_2 used in this study were rutile-type (Table 1). Cyclopentasiloxane (silicone, KF-995, Shin-Etsu Chemical, Co., Tokyo, Japan) was used as the dispersing medium. Purified water and nitric acid used in TiO_2 quantitative analysis were ultra-microanalysis grade from Wako Pure Chemicals Industries, Ltd. (Osaka,

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Table 1. Titanium dioxide used in this study

Abbreviation	Primary particle size*	Coating
T-35	35 nm	uncoated
TC-35	35 nm	alumina · silica · silicone
T-disp	10 nm x 100 nm	mixture of alumina coated and silicone coated
T-250	250 nm	uncoated

All TiO_2 are rutil-type.
* from catalogue of source company.

Japan). A stock solution of titanium containing 1,000 mg/l (Kanto Chemical Co., Inc., Tokyo, Japan) was used to produce standards for calibration curves for Ti analysis. All other chemicals were of reagent grade.

Preparation of suspensions

Drops of silicone were added to a weighed amount of TiO_2 powder in a tube, followed by kneading. Additional silicone was added to bring the concentration of TiO_2 samples to 10%, followed by sonication in a bath-type sonicator (US-3, Iuchi, Tokyo, Japan) for 30 min. The T-disp was diluted with silicone for a final TiO_2 concentration of 10%, followed by sonication.

Evaluation of TiO_2 suspensions

The particle size of TiO_2 in suspension was measured using a dynamic laser scattering apparatus (DLS-8000HL, Otsuka Electrons Co., Osaka, Japan) after a thousand-fold dilution with silicone.

Skin conditions after application of TiO_2 was observed using two methods. Two µl of suspension were applied to an area of skin of approximately 1 cm^2. After drying, the skin surface was observed by digital fine scope microscopy (VC-3000, Omron, Tokyo, Japan) with a magnification of 80. The epidermis of YMP skin prepared by a heat separating method (Kligman and Christophers, 1963) was mounted on a scanning electron microscopy (SEM) stage with adhesive tape. One µl of the suspension were spread over approximately 0.5 cm^2 and dried *in vacuo*. Then, the skin sample was coated with Pt/Pd and examined using SEM (JSM-5200LV, JEOL, 20 kV).

Skin penetration

Yucatan micropig (YMP) skin was used as the model because of its similarity with human skin (Lavker *et al.*, 1991; Fujii *et al.*, 1997). YMP skin (YMP skin set, Charles River Japan, Kanagawa, Japan) removed the subdermal tissue and fat was used as full-thickness skin (intact skin). The SC was removed from intact skin with adhesive tape (Scotch 313, 3M) (stripped skin). Hair was removed from intact skin using tweezers (hair-removed skin).

Two µl of suspension were applied to an area of skin of approximately 1 cm^2. Then the skin was placed on a modified Franz-type diffusion cell. After 24 hr, the receptor phase (pH 7.1 isotonic phosphate buffer solution) was collected, the skin was removed from the diffusion cell and cut off the rim for mounting the cell. Residues on the skin surface were removed by two cyanoacrylate (Aronalfa, Toagosei, Tokyo, Japan) stripping and Ti in the skin was determined. Application amount and period were in accordance with Standard SPF Test Method of Japan Cosmetic Industry Association (1999) and OECD (2004): Test Guideline 428 (skin absorption: *in vitro* method), respectively. For some samples, the epidermis and dermis were separated by heating after cyanoacrylate stripping. A similar procedure was used for obtaining SEM pictures with energy dispersed X-ray spectrometry (SEM-EDS).

Determination of Ti

Approximately 0.1 g of skin or 1 ml of receptor phase was transferred to a Teflon digestion vessel and 5 ml of nitric acid plus 1 ml of purified water was added to each vessel. The vessels were placed in a microwave oven (MARS5, CEM Co., Matthews, NC, USA). The microwave-assisted digestion consisted of increasing the pressure to 80 psi over 20 min and then maintaining that pressure for 20 min by applying 100% power at 1,600 W. For separated epidermis and dermis, approximately 1 cm^2 of skin (*ca.* 0.01 g of epidermis, 0.3 g of dermis) was used. After digestion, the resulting solution was fixed with 20 ml of purified water. The Ti concentration in the samples was measured by ICP-MS (7500, Agilent Technologies, Santa Clara, CA, USA). The amount of Ti was calculated using a standard curve of Ti created with the peak area at mass number 47.

Ti distribution in skin

The skin sample was fixed with Karnovsky solution, dehydrated with ethanol, and replaced with resin. Horizontal cuts were made in the skin from the surface to the dermis and observations were obtained every 50 μm using SEM-EDS (JSM-6700/JED2300, JEOL, Tokyo, Japan).

Statistical analysis

The amount of Ti in skin was determined using at least 3 samples and the data subjected to analysis of variance (ANOVA) followed by Dunnett's test. A value of $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Particle size of TiO_2 in suspension and on the skin surface

Both oil in water (O/W) and water in oil (W/O) creams are used as sunscreen formulations with TiO_2. TiO_2 is usually formulated in the oil phase; the oil suspension using silicone, which is often used for the base of sunscreens because of volatile and repels water characteristics, was used for this study. Many types of TiO_2 particles exist, with differences in crystalline type, size, shape, and surface coating characteristics. Four types of rutile TiO_2 shown in Table 1 was used for this study. T-35 was used to represent typical nanoparticles, with a round shape and no surface coating. TC-35, which has lipophilic coating features, was chosen to produce a good dispersion in silicone. T-disp is a pre-formulated product consisting of TiO_2 dispersed in silicone. The T-250 was used for comparison because it does not form nanoparticles.

Even if the primary particle size is less than 100 nm, lyophobic colloidal dispersions are unstable and aggregation occurs easily. Particle size distributions of TiO_2 in silicone are shown in Fig. 1. Mean particle size of T-35 was 1,700 nm, which was larger than that of T-250, 1,200 nm. Although the possibility exists that large particles shielded small particles, few nanoparticles were found. In contrast, suspensions of TC-35 and T-disp contained nanoparticles with mean diameters of 80 and 130 nm, respectively. The TC-35 suspension contained large particles that were easy to precipitate.

After skin application of suspensions, silicone was spread and vaporized so that only TiO_2 particles remained on the skin. Fig. 2(a) shows microscopic pictures of the surface after application of each suspension to skin followed by drying. T-250 and T-35 suspension showed aggregated white powder. After application of the TC-35 suspension, the skin was covered with white film that was thicker in furrows. Silicone spread easily on the skin and tended to collect in furrows because of low viscosity and interface tension. No particles were observed but the skin was slightly white after application of T-disp suspension. The SEM pictures showed large agglomerated TiO_2 (about 5 μm) for T-35 and T-250, although their primary particle sizes were different. The TC-35 also aggregated into particles of approximately 1 μm, although many small particles stuck to the skin. The T-disp formed uniformly agglomerated particles that differed from other preparations. TiO_2 particles appeared to be covered with dispersing agent (Figs. 2(b), (c)).

Skin penetration of TiO_2

Cosmetics and sunscreens are usually used only on intact skin. However, skin can be injured slightly by objects or through physical force. Thus, skin penetration of TiO_2 was investigated *in vitro* with intact skin and with stripped skin as a model of injured skin. Previous reports have indicated that hair follicles are important in the skin penetration of nanoparticles (Lekki *et al.*, 2007; Zvyagin *et al.*, 2008). Therefore, hair-removed skin was used to represent skin damaged by hair-removal treatments often done for the pursuit of beauty.

After 24-hr application, the skin surface was stripped with cyanoacrylate to remove surface TiO_2. A tape stripping technique is often used to remove residual materials on skin; however, materials in furrows or on hair follicles cannot be removed by this technique (Pflücker *et al.*, 1999). In this study, the amount of Ti varied greatly when

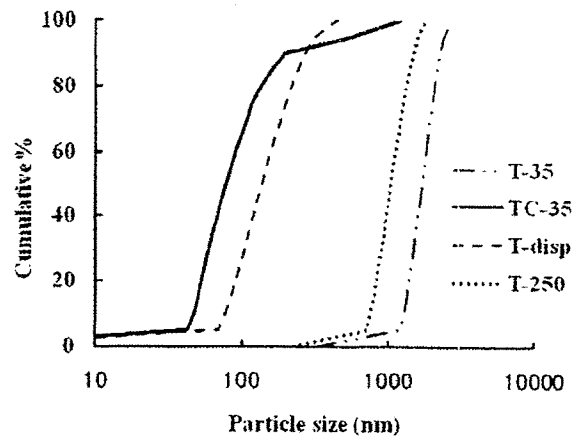

Fig. 1. Particle size of TiO_2 dispersed in silicone. Particle size was measured by dynamic laser scattering using a thousand-fold dilution of 10% TiO_2 silicone suspension.

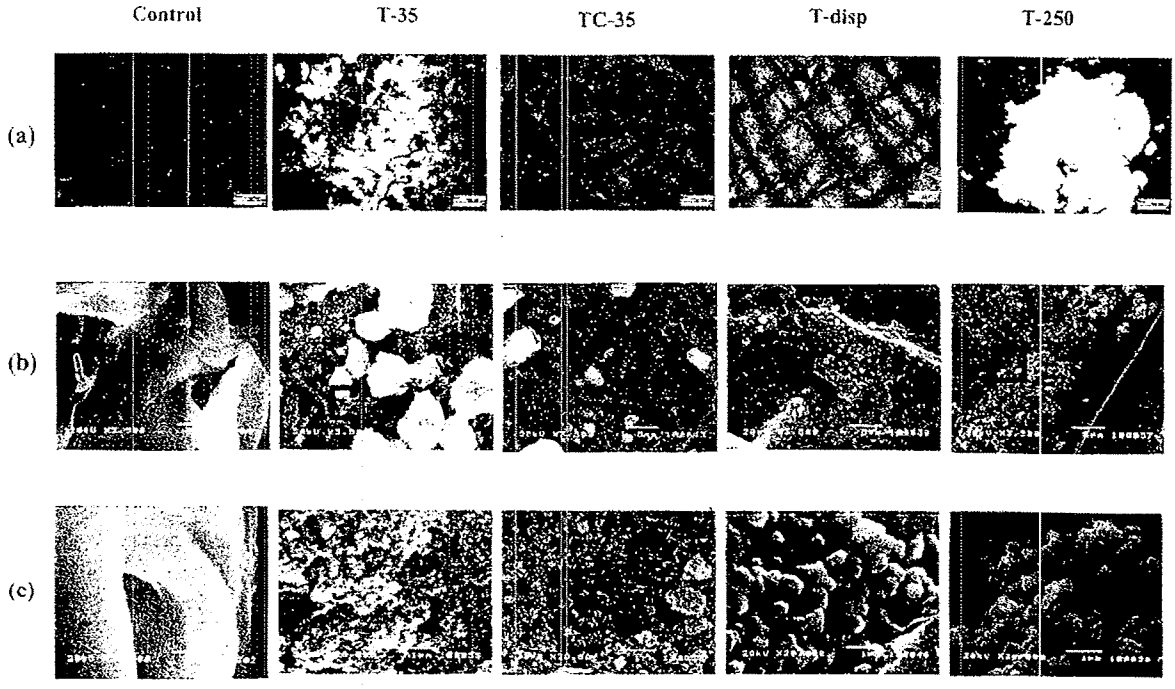

Fig. 2. The conditions of TiO_2 after skin application of a 10% silicon suspension of TiO_2 at a dose of 2 μl/cm². The skin surface was observed using a digital fine scope at a magnification of (a) 80. The surface of the epidermis was observed by SEM at a magnification of (b) 3,500 and (c) 20,000.

Ti remained in furrows (data not shown), so cyanoacrylate stripping was used. In this procedure, the surface SC layer of intact skin and hair-removed skin, and some hairs of intact skin and stripped skin were also removed.

Silicone is used to control formulation properties. Ti concentration in the receptor phase was similar in all skin conditions and formulations applied (Fig. 3(a)). Fig. 3(b) shows Ti concentration values in skin. For intact and stripped skin, no significant difference in Ti concentration was found between the control and suspension applications, which indicates TiO_2 did not penetrated into the skin regardless of particle size and even when the SC, which is the skin's primary barrier, was removed. For hair-removed skin, Ti concentration in skin after application of TC-35 suspension was significantly higher than that of the control, and after application of T-disp suspension, tended to be high. The Ti concentration in the dermis was no different than that of the control. Ti concentration in the epidermis after application of TiO_2 nanoparticles tended to be greater than that of the control, but the difference was not significant (Fig. 4). The epidermis consists of SC, viable epidermis and hair follicles. The horizontal sections from hair-removed skin after application of TC-35 suspension were observed using SEM-EDS. One of two SEM-EDS images showed the presence of Ti in the empty hair follicle after removal of the hair shaft 1 mm from the surface (Fig. 5(a)). Ti was detected in the hair follicle pocket, but not in the surrounding viable skin (Fig. 5(b)). We also found the similar distribution of 20 nm FITC-polystyrene (data not shown). The radius of a hair follicle is 0.05-0.2 mm (Otberg *et al.*, 2004), which would allow solvent to enter if the hair shaft and sebum did not fill the follicle space. When fluid enters a small space by capillary action, small particles of Ti in fluid may be able to enter the follicle. Large particles cannot be moved by such a small force, but TC-35 well dispersed in solvent might enter a follicle more easily than other types of TiO_2. For T-disp, the dispersing agent had some effect, resulting in particles left on the skin after drying of the suspension.

In conclusion, TiO_2 does not penetrate into viable skin, even if the particle size is less than 100 nm and the SC is damaged. However, immediately after hair removal, some TiO_2 particles penetrated relatively deeply into the

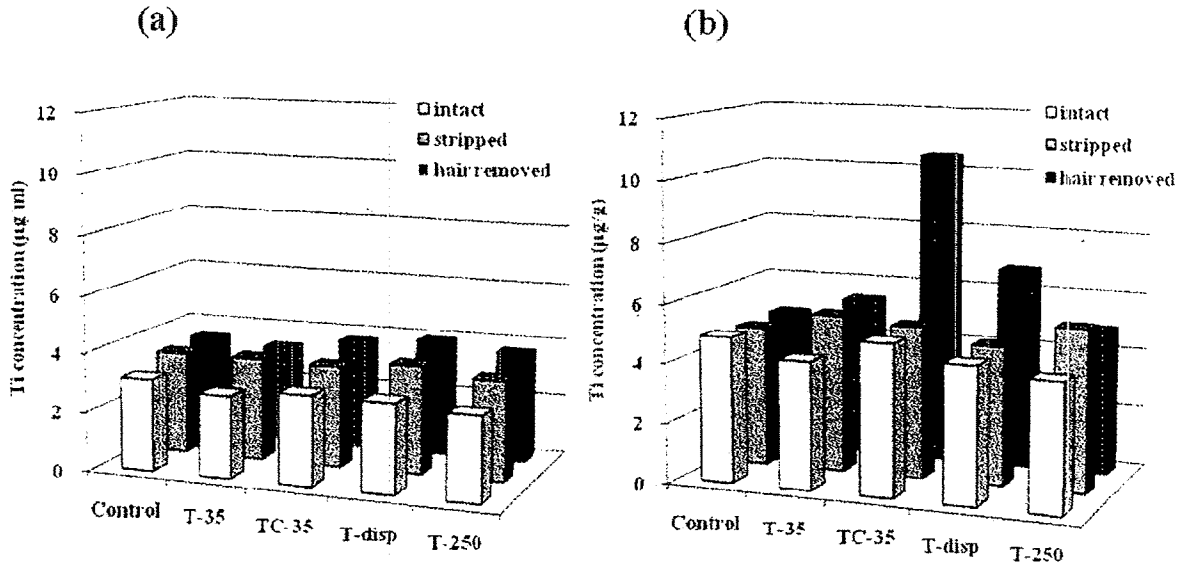

Fig. 3. Ti concentration in receptor phase (a) and in skin (b) after 24 hr application of a 10% silicone suspension of TiO_2 at a dose of 2 μl/cm² on intact skin, stripped skin, and hair-removed skin.
Silicon applied as a control. TiO_2 on the skin surface was removed by cyanoacrylate stripping.

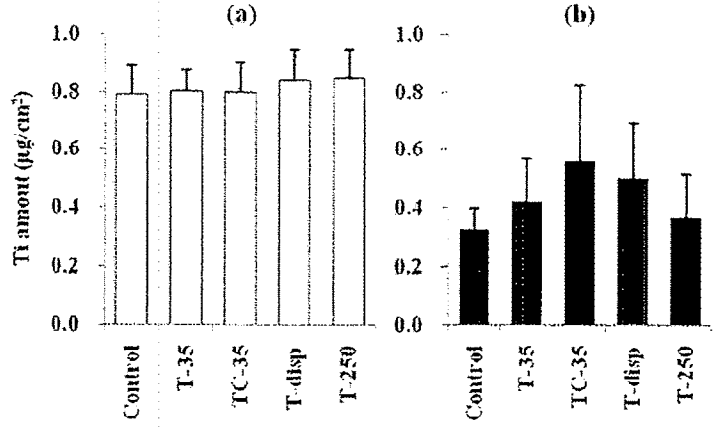

Fig. 4. Amount of Ti in (a) dermis and (b) epidermis after 24 hr application of a 10% silicone suspension TiO_2 at a dose of 2 μl/cm² to hair-removed skin.
Silicone was applied as a control. TiO_2 on the skin surface was removed by cyanoacrylate stripping, followed by separation of the dermis and epidermis by heat.

skin, possibly by entering the empty hair follicle. This was an *in vitro* study, so no inflammation was induced by the hair removal procedure. Inflammation could affect these results, therefore, further *in vivo* studies on viable skin with the hair removed are necessary.

There might be various nanoparticls to determine skin penetration, thus *in vitro* method is important for screening. If the materials have possibility to use on hair

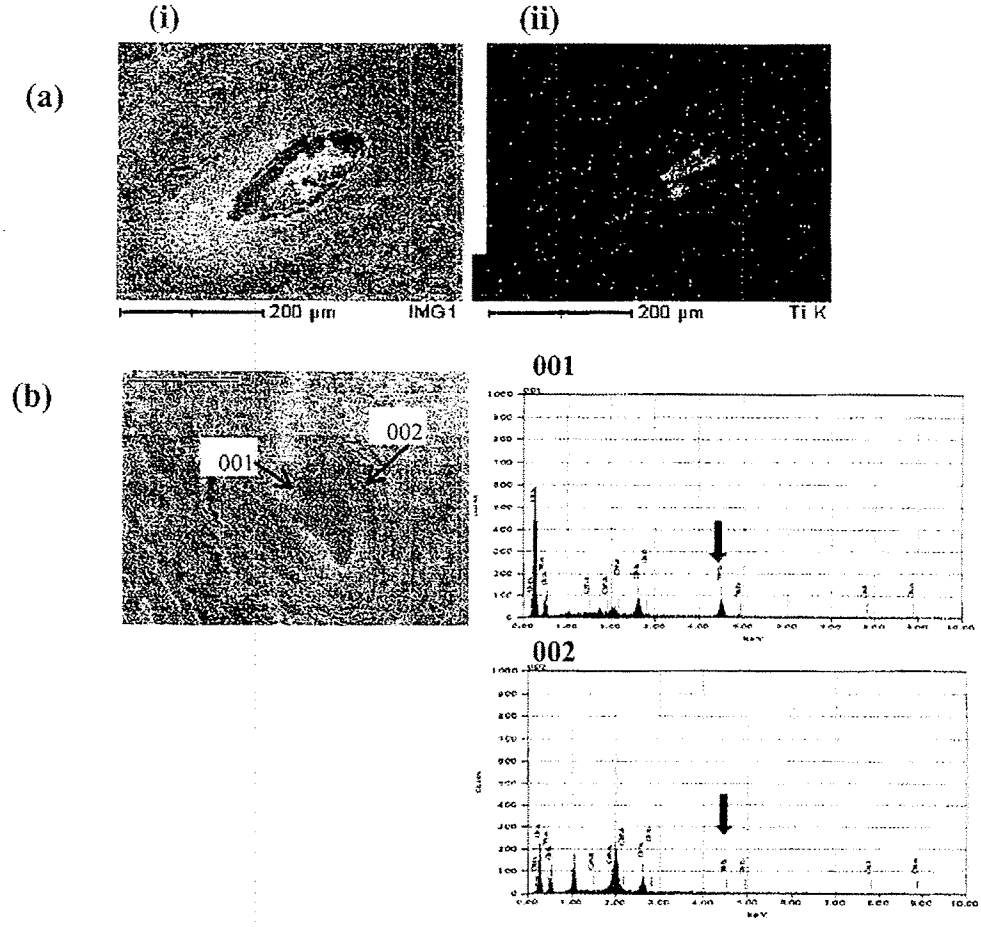

Fig. 5. SEM-EDS images (a) and elemental analysis (b) of a horizontal section of skin after 24 hr application of a 10% silicone suspension of TC-35 at a dose of 2 μl/cm² to hair-removed skin.
(a) at a depth of 1,050 μm from the skin surface. (i) SEM images, (ii) Ti distribution; (b) at a depth of 1,250 μm from the skin surface, (001) the hair follicle, (002) the dermis in contact with the hair follicle.

removed skin, hair removed skin should be taken into safety assessment of nanoparticles as well as stripped skin as a model of damaged skin. Also, this results indicate that the split skin (thickness 200~400 μm) which OECD Test guideline 428 recommended for skin absorption study *in vitro* have possibility to overestimate the skin permeation of nanoparticles because hair follicle is cut and nanoparticle in hair follicle is into receptor phase.

ACKNOWLEDGMENTS

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Research paper

Anticarcinogenesis pathways activated by bovine lactoferrin in the murine small intestine

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Abstract

Oral administration of bovine lactoferrin (bLF) inhibits carcinogenesis in the colon and other organs in rats, and lung metastasis in mice. A likely mechanism by which bLF mediates its anticarcinogenesis effects is by enhanced expression of cytokines and subsequent activation of immune cells. Oral administration of bLF enhances expression of interleukin-18 (IL-18) mRNA in the mucosa of the small intestine of mice. Importantly, the pepsin hydrolysate of bLF (bLFH) also induced expression of IL-18 mRNA in the mouse small intestine and a peptide produced by pepsin digestion of bLF, bovine lactoferricin (bLFcin), induced expression of mature IL-18 in organ culture. In addition to IL-18, bLF and bLFcin both induced significant increases in caspase-1 activity in peritoneal macrophages and in organ cultures. The increase of mature IL-18 by macrophages was inhibited by caspase-1 inhibitor: caspase-1 is known to cleave the proform of IL-18 to produce active mature IL-18. Finally, bLF also induced expression of IFNγ by peritoneal macrophages. Importantly, in IFNγ knockout (GKO) mice, bLF administration resulted in increased expression of caspase-1 protein, but induction of IL-18 mRNA, caspase-1 activity, and mature IL-18 was not observed. These results indicate that orally administered bLF can induce expression of IFNγ and caspase-1 in the small intestine. IFNγ in turn increases expression of target genes, including IL-18. Active caspase-1 then cleaves pro-IL-18 to generate mature IL-18. Thus, bLF activates an effector pathway mediated by IFNγ, caspase-1, and IL-18. We also show that ingested bLF is able to activate more than a single effector pathway. For example, in GKO mice while bLF administration could not activate the IFNγ/caspase-1/IL-18 effector pathway, it was able to inhibit tumor growth and metastasis by activation of an IFNα/IL-7 effector pathway.

Keywords: Lactoferrin; Innate immune system; Cytokine; Chemoprevention; Carcinogenesis

1. Introduction

Lactoferrin (LF), an approximately 80 kDa iron binding glycoprotein initially purified from milk, is an important component of the innate immune system. A potent antimicrobial and antiviral agent [1–5], LF is a major component of antimicrobial host defense and is found in a variety of exocrine secretions, e.g., tears, nasal exudate, saliva, bronchial mucus, gastrointestinal fluids, cervicovaginal mucus, and seminal fluid. In addition to its presence in mucosal secretions, LF is released by activated neutrophils at septic sites.

The concentration of LF in human colostrum is particularly high (7–10 mg/ml) [6] resulting in ingestion of 1–2 g/day for several months by a suckling infant [7]. It is thought that orally ingested LF may be able to interact with epithelial cells and immune cells in the mucosa of the intestine: ingested LF is reported to stimulate cytokine

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