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トランスクリプトーム解析を利用した医薬品の副作用発症機構の解明と、それに基づいた副作用予測システム、副作用治療法、及び副作用の少ない新薬の開発戦略の確立

平成 25 年度　総括研究報告書

研究代表者　水島　徹

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目次

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トランスクリプトーム解析を利用した医薬品の副作用発症機構の解明と、それに基づいた副作用予測システム、副作用治療法、及び副作用の少ない新薬の開発戦略の確立

研究代表者　水島　徹　慶應義塾大学薬学部教授

研究要旨

平成２５年度我々は、小柴胡湯（間質性肺炎を起こす漢方薬）解析を行った。その結果、小柴胡湯が活性酸素の産生酵素である NADPH オキシダーゼを活性化すること、及び抗酸化タンパク質である SOD の発現を抑制することを見出した。また、これらの抑制がこれら医薬品による間質性肺炎の原因であることを示唆した。

一方我々は、保有する既存薬ライブラリーから SOD の発現を抑制するものを検索し、それらがマウスで肺線維化を起こすのかを検討した。その結果、複数の既存薬が SOD の発現を抑制すると同時に肺の線維化を起こすことを見出し、その内一部に関しては、薬剤性間質性肺炎を起こしたという臨床報告があった。以上の結果は、新薬候補品が SOD の発現を抑制するのかを調べることにより、その間質性肺炎副作用を予測することが可能であることを提唱した。

さらに横紋筋融解症が問題になっている抗脂血症薬、薬剤性過敏症（薬疹）が問題になっている抗てんかん薬、スティーブンス・ジョンソン症候群が問題になっている抗生物質に関して、当該副作用発症機構を解明するために、それぞれの薬を細胞に作用させ、そこでおこる遺伝子発現変化を現在解析している。

A. 研究目的

既存薬による副作用発症機構が理解されていないため、基礎研究段階で新薬候補品の副作用を予測出来ずに（臨床試験で初めて副作用が発見され）、臨床試験が失敗している。そこで本研究で我々は、トランスクリプトーム解析を基に医薬品の副作用発症機構を解明し、新薬候補品の副作用を予測するシステムを確立する。また、副作用の少ない新薬の開発や副作用治療法の確立も目指す。以下に、我々のこれまでの研究成果を述べる。

アスピリンを代表とするNSAIDは優れた抗炎症薬として世界中でよく使用されているが、その胃潰瘍副作用（NSAID潰瘍）が臨床現場で大きな問題になっている（米国では年間16500人が亡くなっている）。我々はNSAIDが誘導する遺伝子を網羅的に解析し（トランスクリプトーム解析）、NSAIDが膜傷害性を持つこと、及びこれがNSAID潰瘍の原因であることを見出した。この成果を受けて製薬企業は、膜傷害性を指標に新薬候補品の胃潰瘍副作用を予測するスクリーニングを開始している。また我々は、膜傷害性の少ないNSAIDの合成に世界で初めて成功し、それらが充分な抗炎症作用を示すにも関わらず、ほとんど胃潰瘍を起こさないことを見いだした（現在、製薬企業で開発中）。

また我々は、間質性肺炎副作用が問題になっているレフルノミドに関しても、トランスクリプトーム解析を行った。その結果、レフルノミドが上皮間葉転換（EMT）を起こすこと、及びこれが間質性肺炎副作用の原因であることを明らかにした。一部の製薬企業では、EMT誘導を指標に新薬候補品の間質性肺炎副作用を予測するスクリーニングを開始している。また我々は、このEMT誘導を抑制する薬剤の肺内投与が、レフルノミド依存のマウス間質性肺炎を抑制することを見出した（副作用治療法の確立に繋がる成果）。

以上の成果を受けて本研究で我々は、他の薬剤による間質性肺炎副作用（平成23-24年度実施）、及び薬疹など他の副作用（平成24-25年度実施）に関して、トランスクリプトーム解析を用いて副作用発症機構を解明し、新薬候補品の副作用を予測するシステムを確立すると共に、副作用の少ない新薬の開発、及び副作用治療法の確立も目指す。

B. 研究方法

（1）ゲフィチニブの間質性肺炎副作用に関する研究

ゲフィチニブ（イレッサ）の間質性肺炎（肺繊維症）副作用による死亡者は多く、社会問題になっている。一方、ある種の肺癌治療にはこの医薬品が必要不可欠であり、その治療法の確立、及び副作用の少ないゲフィチニブ誘導体（改良薬）の開発が急務になっている。

最近我々は、ゲフィチニブによる遺伝子発現変化の網羅的解析から、ゲフィチニブが熱ショックタンパク質（HSP）70（強力な細胞保護作用と抗炎症作用を持つ）の発現を強く抑制することを発見した。また我々はマウスを用いて、ゲフィチニブ依存に肺繊維化を起こす系（薬剤性間質性肺炎の動物モデル）を確立し、このモデルにおいてゲフィチニブ依存にHSP70の発現が抑制されること、及びHSP70過剰発現マウス（ゲフィチニブによるHSP70発現抑制が起こらないマウス）では、ゲフィチニブ依存の肺繊維化も見られないことを見出した。以上の結果は、ゲフィチニブはHSP70の発現を抑制することにより、間質性肺炎（肺繊維症）を起こすことを示唆している。そこで以下に述べる研究を行う。

①HSP70に着目した新薬候補品の間質性肺炎副作用予測システムの確立（平成23年度実施）

我々が保有する既存薬ライブラリーからHSP70の発現を抑制するものを検索し、それらがマウスで肺線維化を起こすかを検討した。肺線維化を起こした既存薬（10数種）に関して、症例報告や副作用データベースを用いて、間質性肺炎副作用の有無を調べた。その結果、複数の既存薬に関して間質性肺炎副作用報告があり、HSP70発現抑制作用を調べることが間質性肺炎副作用の予測システムとして有用であることを示唆した。今後、製薬企業にこのシステムの導入を促す（目標：少なくとも3社）。

②HSP70誘導薬による、ゲフィチニブ依存性間質性肺炎治療法の確立（平成23年度実施）

上述の結果は、HSP70 誘導薬がゲフィチニブ依存の間質性肺炎治療に有効であることを示唆している。我々は日本で最もよく使われている胃薬・ゲラニルゲラニルアセトン（GGA、商品名セルベックス）が HSP70 を誘導することを報告している（***JBC***, 2007, 2009, 2010 など）。そこで我々が確立した動物モデルを用いて、ゲフィチニブ依存性間質性肺炎治療薬としての GGA の有効性を検討したところ、GGA 投与によりゲフィチニブ依存の肺繊維化、及び HSP70 の発現抑制が見られなくなることを見出した。今後臨床研究へ繋げるための準備を行う。（GGA は既に臨床で使われているので、すぐに臨床研究を行うことが出来る）。（目標：25 年度中の臨床研究開始）

③間質性肺炎副作用の少ないゲフィチニブ誘導体の発見（平成 24 年度実施予定）

数多くのゲフィチニブ誘導体を合成しその中から、試験管内で HSP70 発現抑制効果がなく、かつゲフィチニブと同程度の癌細胞増殖抑制効果を有するものを選択する。次に動物実験を行い、ゲフィチニブと同程度の抗癌作用を持ち、かつ肺繊維化を起こさないものを選択する。特許を取得したのち、間質性肺炎副作用の少ないゲフィチニブ改良薬としての開発を製薬企業へ提案する。（目標：25 年度中の特許出願）。

（２）他の薬剤性間質性肺炎に関する研究（平成 24 年度実施予定）。

レフルノミドやゲフィチニブ以外にも、抗癌剤（イマチニブなど）、抗リウマチ薬（メトトレキサートなど）、漢方薬（小紫胡湯など）が間質性肺炎を起こすことが知られているが、その発症機構は分かっていない。そこで、これらの薬剤による遺伝子発現変化の網羅的解析（トキシコゲノミックス・データベース等を利用する）からその副作用発症機構を解明する。

また上述のゲフィチニブの場合と同様の方法で、新薬候補品の副作用を予測するシステムの確立、副作用治療法の確立、副作用の少ない誘導体の発見を目指す。（目標：少なくとも２薬剤の副作用機構の解明）

（３）他の副作用に関する研究（平成 25 年度実施予定）

抗脂血症薬による横紋筋融解症、抗てんかん薬による薬剤性過敏症（薬疹）、抗生物質によるスティーブンス・ジョンソン症候群、糖尿病薬による肝障害などに関しても、副作用発症機構を解明し、新薬候補品の副作用を予測するシステムを確立すると共に、副作用治療法の開発、及び副作用の少ない誘導体の発見を目指す。（目標：少なくとも２薬剤の副作用機構の解明）

C. 研究結果

平成２５年度我々は、小柴胡湯（間質性肺炎を起こす漢方薬）解析を行った。その結果、小柴胡湯が活性酸素の産生酵素であるNADPHオキシダーゼを活性化すること、及び抗酸化タンパク質であるSODの発現を抑制することを見出した。また、これらの抑制がこれら医薬品による間質性肺炎の原因であることを示唆した。

一方我々は、保有する既存薬ライブラリーからSODの発現を抑制するものを検索し、それらがマウスで肺線維化を起こすのかを検討した。その結果、複数の既存薬がSODの発現を抑制すると同時に肺の線維化を起こすことを見出し、その内一部に関しては、薬剤性間質性肺炎を起こしたという臨床報告があった。以上の結果は、新薬候補品がSODの発現を抑制するのかを調べることにより、その間質性肺炎副作用を予測することが可能であることを提唱した。

さらに横紋筋融解症が問題になっている抗脂血症薬、薬剤性過敏症（薬疹）が問題になっている抗てんかん薬、スティーブンス・ジョンソン症候群が問題になっている抗生物質に関して、当該副作用発症機構を解明するために、それぞれの薬を細胞に作用させ、そこでおこる遺伝子発現変化を現在解析している。

D.考察

結果の欄に記載した。

E.結論

結果の欄に記載した。

F.健康危険情報

該当なし

G.研究発表

1. 論文発表

1. Nakashita, M., Suzuki, H., Miura, S., Taki, T., Uehara, K., Mizushima, T., Nagata, H. and Hibi, T. Attenuation of acetic acid-induced gastric ulcer formation in rats by glucosylceramide synthase inhibitors. ***Dig. Dis. Sci.*** 58, 354-362. (2013)
2. Matsuda, M., Hoshino, T., Yamakawa, N., Tahara, K., Adachi, H., Sobue, G., Maji, D., Ihn, H. and Mizushima, T. Suppression of UV-induced wrinkle formation by induction of HSP70 expression in mice. ***J. Invest. Dermatol.*** 133, 19-28. (2013)
3. Yamakawa, N., Suemasu, S., Watanabe, H., Tahara, K., Tanaka, K., Okamoto, Y., Ohtsuka, M., Maruyama, T. and Mizushima, T. Comparison of pharmacokinetics between loxoprofen and its derivative with lower ulcerogenic activity, fluoro-loxoprofen. ***Drug Metab. Pharmacokinet.*** 28, 118-124. (2013).
4. Tanaka, R., Watanabe, H., Kodama, A., Chuang, VTG., Ishima, Y., Hamasaki, K., Tanaka, K, Mizushima, T., Otagiri, M. and Maruyama, T. Long-acting human serum albumin-thioredoxin fusion protein suppresses bleomycin-induced pulmonary fibrosis progression. ***J. Pharmacol. Exp. Ther.*** 345, 271-283. (2013).
5. Ishihara, T., Yamashita, Y., Takasaki, N., Yamamoto, S., Hayashi, E., Tahara, K., Takenaga, M., Yamakawa, N., Ishihara, T., Kasahara, T. and Mizushima, T. Prostaglandin E_1-encapsulated nanoparticles improve walking activity in an experimental rat model of intermittent claudication. ***J. Pharm. Pharmacol.*** 65, 1187-1194. (2013).
6. Yamashita, Y., Hanaya, K., Sugai, T., Mizushima, T. and Shoji, M. Chemo-enzymatic enantioconvergent approach toward ethyl shikimate from ethyl 5-hydroxy-3,4-isopropylidenedioxycyclohex-1-enecarboxylate. ***Tetrahedron*** 69, 6527-6532. (2013).
7. Hoshino, T., Suzuki, K., Matsushima, T., Yamakawa, N., Suzuki, T. and Mizushima, T. Suppression of Alzheimer's disease-related phenotypes by geranylgeranylacetone in mice. ***PLoS One*** 8, e76306. (2013).
8. Tanaka, K., Ishihara, T., Sugizaki, T., Kobayashi D., Yamashita, Y., Tahara, K., Yamakawa, N., Iijima, K., Mogushi, K., Tanaka, H., Sato, K., Suzuki, H. and Mizushima, T. Mepenzolate bromide displays beneficial effects in a mouse model of chronic obstructive pulmonary disease. ***Nature Communications*** 4, 2686. (2013).
9. Yamakawa, N., Suzuki, K., Yamashita, Y., Katsu, T., Hanaya, K., Shoji, M., Sugai, T. and Mizushima, T. Structure-activity relationship of celecoxib and rofecoxib for the membrane permeabilizing activity. ***Bioorg. & Medic. Chem.*** 22, 2529-2534. (2014).
10. Tanaka, K., Kurotsu, S., Asano, T., Yamakawa, N., Kobayashi D., Yamashita, Y., Yamazaki, H., Ishihara, T., Watanabe, H., Maruyama, T., Suzuki, H. and Mizushima, T. Superiority of pulmonary administration of mepenzolate bromide over other routes as treatment for chronic obstructive pulmonary disease. ***Sci. Rep.*** 28, 4510. (2014).
11. Sawazaki, R., Ishihara, T., Usui, S., Hayashi, E., Tahara, K., Hoshino, T., Higuchi, A., Nakamura, S., Tsubota, K. and Mizushima, T. Diclofenac protects cultured human corneal epithelial cells against hyperosmolarity and ameliorates corneal surface damage in a rat model of dry eye. ***Invest. Ophthalmol. Vis. Sci.*** 55, 2547-2556. (2014).

2. 学会発表（招待講演のみ）

1 水島徹　レシチン化 SOD の開発 東京大学大学院新領域研究科での招待講演 (2013)（柏）

2 水島徹　ドラッグリポジショニングとは何か、新薬開発にどのような道が開けるのか レギュラトリーサイエンス エキスパート研修会での招待講演 (2013)（東京）

3 水島徹　ドラッグリポジショニング-既存薬を利用した新薬開発- 第 55 回鹿児島消化器病研究会での特別講演 (2013)（鹿児島）

4 水島徹　PC-SOD 吸入製剤の開発 日本薬学会シンポジウムでの招待講演 (2013)（横浜）

5 水島徹　ストレスから体を守るタンパク質・HSP の働きと、その医薬品・
化粧品への応用 榊原記念病院定例講演会での特別講演 (2013)（東京）

6 水島徹　熱ショックタンパク質（HSP）の機能とその応用 第 29 回動物植物工学シンポジウムでの招待講演 (2013)（東京）

7 水島徹　ヒートショックプロテイン(HSP)と分子シャペロン 第 56 回センサーネットワーク研究会特別講演 (2013)（東京）

8 水島徹　ドラッグリポジショニング（DR）の現状と今後の展望 日本バイオインダストリー協会での招待講演 (2013)（東京）

9 ドラッグリポジション(DR)の考えに基づく新規アルツハイマー治療薬の開発 学術会議シンポジウム「リバーストランスレーショナルリサーチ」での招待講演 (2013)（東京）

10 水島徹　ドラッグリポジショニングに関するイントロダクション 日本バイオインダストリー協会での招待講演 (2013)（東京）

11 水島徹　ドラッグリポジショニング-既存薬の新しい作用機構の発見と適応拡大- 感染病態研究フロンティアでの特別講演 (2013)（東京）

12 水島徹　臨床試験に直結する DDS 研究を目指して 第 29 回日本 DDS 学会での受賞講演 (2013)（京都）

13 水島徹　人の命を救う新薬を薬学部から世界へ DR と DDS による 21 世紀型新薬開発戦略の提案 崇城大学薬学部での招待講演 (2013)（熊本）

14 水島徹　慶應ネットワークによるドラッグリポジショニング創薬研究シンポジウム-医工薬連携による慶應初創薬を目指して-での招待講演 (2013)（東京）

15 水島徹　慶應ネットワークによるドラッグリポジショニング創薬研究シンポジウム-医工薬連携による慶應初創薬を目指して-での招待講演 (2013)（東京）

16 Tohru Mizushima Scientist as a pharmacist, Pharmacist as a Scientist. Invited lecture in 12th Asia Pacific Pharmaceutical Symposium in JAPAN. (2013) (Tokyo)

17 水島徹　HSP によるシミ・シワ予防 GGA・HSP 勉強会での招待講演 (2013)（東京）

18 水島徹　美容・健康で活用できる HSP の知識 ダイエット＆ビューティーフェア 2013 での招待講演 (2013)（東京）

19 水島徹　HSP の様々な機能とドラッグリポジショニング 日本生化学会シンポジウムでの招待講演 (2013)（横浜）

20 水島徹　胃潰瘍を起こさない NSAID の開発 日本線維筋痛症学会シンポジウムでの招待講演 (2013)（横浜）

21 水島徹　ドラッグリポジショニングの現状と展望 生命医薬情報学連合大会シンポジウムでの招待講演 (2013)（東京）

22 水島徹　非ステロイド抗炎症薬の抗アルツハイマー病効果の分子機構と創薬 日本認知症学会シンポジウムでの招待講演 (2013)（松本）

23 水島徹　ドラッグリポジショニング--既存薬の新しい作用機構の発見と適応拡大-- 日本ストレス学会での特別講演 (2013)（徳島）

24 水島徹　ドラッグリポジショニング--既存薬の新しい作用機構の発見と適応拡大—持田製薬研

究所での特別講演　(2013)（御殿場）

25 Tohru Mizushima Drug repositioning Case Study for COPD. Invited lecture in BIT's 1st Annual International Symposium of Drug Repositioning-2013 (2013) (Haikou)

26 水島徹　ドラッグリポジショニング--既存薬の新しい作用機構の発見と適応拡大--　医薬品医療機器レギュラトリーサイエンス財団、医薬品品質分野 次世代リーダー育成研修講座での招待講演　(2013)（東京）

27 水島徹　ドラッグリポジショニングの現状と課題　日本分子生物学学会シンポジウムの招待講演　(2013)（神戸）

28 水島徹　潰瘍学を考える　日本潰瘍学会シンポジウムの招待講演　(2013)（大阪）

29 水島徹　人の命を救う薬を作ろう　熊本学園大学付属高校での特別講演　(2013)（熊本）

30 水島徹　リポジショニングによる医薬品開発　公益財団法人ヒューマンサイエンス振興財団での招待講演　(2013)（東京）

31 水島徹　安く早く確実に安全な医薬品を開発する戦略ドラッグリポジショニング　富山大学・生命融合科学によるファーマ・メディカルエンジニアリングシンポジウムでの招待講演 (2013)（富山）

32 水島徹　NSAID潰瘍の発症機構とその対策　日本消化器内視鏡学会関東地方会での招待講演 (2013)（東京）

33 水島徹　ドラッグリポジショニング　JST-ERATO河岡感染宿主応答ネットワークプロジェクトシンポジウムでの招待講演 (2014)（東京）

34 水島徹　ドラッグリポジショニング　第2回 TR シンポジウムでの招待講演　(2014)（東京）

35 水島徹　熱ショックタンパク質の機能とその応用　豊橋創造大学での招待講演　(2014)（豊橋）

36 水島徹　ドラッグリポジショニングの現状と課題　千里ライフサイエンス専門実務セミナー・新規効能治療薬の創製「ドラッグ・リポジショニングを用いて」での基調講演　(2014)（大阪）

37 水島徹　分子シャペロンと創薬 Diabetes Scientific Seminar での特別講演　(2014)（東京）

38 水島徹　ドラッグリポジショニングの現状と課題　日本薬学会シンポジウムでの招待講演 (2014)（熊本）

39 Tohru Mizushima Identification of a unique NSAID, fluoro-loxoprofen with gastroprotective activity. Invited lecture in the 4th International Forum in Annual Meeting of the Japanese Society of Gastroenterology. (2014) (Tokyo)

40 Tohru Mizushima Therapeutic effect of lecithinized superoxide dismutase (PC-SOD) on idiopathic pulmonary fibrosis in humans and bleomycin-induced pulmonary fibrosis in mice. Invited lecture in the International Advanced Drug Delivery Symposium. (2014) (Taipei)

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

3.その他

該当なし

研究成果の刊行に関する一覧表

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
Nakashita, M., Suzuki, H., Miura, S., Taki, T., Uehara, K., Mizushima, T., Nagata, H. and Hibi, T.	Attenuation of acetic acid-induced gastric ulcer formation in rats by glucosylceramide synthase inhibitors.	***Digestive Diseases and Sciences***	58	***354-362.***	2013
Matsuda, M., Hoshino, T., Yamakawa, N., Tahara, K., Adachi, H., Sobue, G., Maji, D., Ihn, H. and Mizushima, T.	Suppression of UV-induced wrinkle formation by induction of HSP70 expression in mice	***J. Invest. Dermatol***	133	19-28	2013
Yamakawa, N., Suemasu, S., Watanabe, H., Tahara, K., Tanaka, K., Okamoto, Y., Ohtsuka, M., Maruyama, T. and Mizushima, T.	Comparison of pharmacokinetics between loxoprofen and its derivative with lower ulcerogenic activity, fluoro-loxoprofen.	***Drug Metab. Pharmacokinet.***	28	118-124	2013
Tanaka, R., Watanabe, H., Kodama, A., Chuang, VTG., Ishima, Y., Hamasaki, K., Tanaka, K, Mizushima, T., Otagiri, M. and Maruyama, T.	Long-acting human serum albumin-thioredoxin fusion protein suppresses bleomycin-induced pulmonary fibrosis progression.	***Biochem. Pharmacol.***	345	271-283.	2013
Ishihara, T., Yamashita, Y., Takasaki, N., Yamamoto, S., Hayashi, E., Tahara, K., Takenaga, M., Yamakawa, N., Ishihara, T., Kasahara, T. and Mizushima, T.	Prostaglandin E_1-encapsulated nanoparticles improve walking activity in an experimental rat model of intermittent claudication.	***Am. J. Physiol. Lung Cell. Mol. Physiol.***	65	1187-1194	2013
Yamashita, Y., Hanaya, K., Sugai, T., Mizushima, T. and Shoji, M.	Chemo-enzymatic enantioconvergent approach toward ethyl shikimate from ethyl 5-hydroxy-3,4-isopropylidenedioxycyclohex-1-enecarboxylate.	***Tetrahedron***	69	6527-6532	2013
Hoshino, T., Suzuki, K., Matsushima, T., Yamakawa, N., Suzuki, T. and Mizushima, T.	Suppression of Alzheimer's disease-related phenotypes by geranylgeranylacetone in mice.	***PLoS One***	8	e76306	2013
Tanaka, K., Ishihara, T., Sugizaki, T., Kobayashi D., Yamashita, Y., Tahara, K., Yamakawa, N., Iijima, K., Mogushi, K., Tanaka, H., Sato, K., Suzuki, H. and Mizushima, T.	Mepenzolate bromide displays beneficial effects in a mouse model of chronic obstructive pulmonary disease.	***Nature Communications***	4	2686	2013

Yamakawa, N., Suzuki, K., Yamashita, Y., Katsu, T., Hanaya, K., Shoji, M., Sugai, T. and Mizushima, T.	Structure-activity relationship of celecoxib and rofecoxib for the membrane permeabilizing activity.	***Bioorg. & Medic. Chem.***	22	2529-2534	2014
Tanaka, K., Kurotsu, S., Asano, T., Yamakawa, N., Kobayashi D., Yamashita, Y., Yamazaki, H., Ishihara, T., Watanabe, H., Maruyama, T., Suzuki, H. and Mizushima, T.	Superiority of pulmonary administration of mepenzolate bromide over other routes as treatment for chronic obstructive pulmonary disease.	***Scientific Reports***	28	4510	2014
Sawazaki, R., Ishihara, T., Usui, S., Hayashi, E., Tahara, K., Hoshino, T., Higuchi, A., Nakamura, S., Tsubota, K. and Mizushima, T.	Diclofenac protects cultured human corneal epithelial cells against hyperosmolarity and ameliorates corneal surface damage in a rat model of dry eye.	***Invest. Ophthalmol. Vis. Sci.***	55	2547-2556	2014

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ORIGINAL ARTICLE

Attenuation of Acetic Acid-Induced Gastric Ulcer Formation in Rats by Glucosylceramide Synthase Inhibitors

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Abstract
Introduction Ceramide has been suggested to play a role in apoptosis during gastric ulcerogenesis. The present study is designed to investigate whether accumulated ceramide could serve as the effector molecules of ulcer formation in a rat model of acetic acid-induced gastric ulcer.
Methods The effect of fumonisin B1, an inhibitor of ceramide synthase, and of *d,l,-threo*-1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol (PPMP) and N-butyldeoxynojirimycin (NB-DNJ), both inhibitors of glucosylceramide synthase, on the accumulation of ceramide and formation of gastric ulcer were examined in the rat model of acetic acid-induced gastric ulcer.
Results Fumonisin B1 attenuated acetic acid-induced gastric ulcer formation, associated with a decrease in the number of apoptotic cells. Our results showed that it is neither the C18- nor the C24-ceramide itself, but the respective metabolites that were ulcerogenic, because PPMP and NB-DNJ attenuated gastric mucosal apoptosis and the consequent mucosal damage in spite of their reducing the degradation of ceramide.
Conclusion The ceramide pathway, in particular, the metabolites of ceramide, significantly contributes to acetic acid-induced gastric damage, possibly via enhancing apoptosis. On the other hand, PPMP and NB-DNJ treatment attenuated gastric mucosal apoptosis and ulcer formation despite increasing the ceramide accumulation, suggesting that it was not the ceramides themselves, but their metabolites that contributed to the ulcer formation in the acetic acid-induced gastric ulcer model.

Keywords Ceramide · Glucosylceramide inhibitor · Gastric ulcer · Acetic acid · Apoptosis

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Introduction

While many factors have been thought to be involved in the pathogenesis of gastric ulcers, the mechanism of ulcer formation is not yet precisely understood. Gastric mucosal apoptosis is known to be associated with the loss of mucosal integrity and may play an important role in ulcer development [1, 2]. Recently, enhanced apoptosis in the gastric epithelium has been demonstrated to be of pathophysiological importance in various kinds of gastric lesions, such as stress-induced ulcers [1], *Helicobacter pylori*-positive ulcers [3–5], nonsteroidal anti-inflammatory drug (NSAID)-induced ulcers [6], and chemically induced ulcers, such as ethanol-induced ulcers [7, 8]. Inflammatory cytokines, including tumor necrosis factor (TNF)-α and interferon (IFN)-γ, have been postulated to play a role in gastric mucosal apoptosis [8].

Recent studies have revealed that sphingolipids (ceramide, sphingosine, etc.) are highly bioactive compounds that are involved in diverse cell processes, including cell-to-cell interactions, adhesion, differentiation and oncogenic transformation [9], as well as cell proliferation and apoptosis. Accumulation of the sphingolipid ceramide (Cer) is a well-known phenomenon in cells undergoing apoptosis [10, 11], and ceramide analogues have been reported to induce apoptosis [12]. In addition to their direct action on apoptosis, ceramides have also been suggested to have a role in apoptosis induced by the addition of extracellular agents, such as TNF-α [13, 14], IFN-γ [15] or the anti-Fas antibody [16].

Ceramide analogues have been demonstrated in vitro to induce apoptosis in gastric mucosal cell lines. We previously reported that the subserosal injection of phorbol-12-myristate-13-acetate (PMA) resulted in the formation of gastric ulcers in the rat gastric mucosa [17], associated with a significant increase in the cellular contents of ceramides (C18 and C24 ceramide) [18]. The significant ceramide accumulation was thought to have contributed to the PMA-induced tissue damage in that rat model, possibly via enhancing the apoptotic activity in the gastric mucosa, because co-administration of caspase inhibitors or an inhibitor of sphingolipid biosynthesis attenuated the formation of the gastric ulcers, associated with a reduction in the number of apoptotic cells [18]. However, it remains unknown whether the ceramide-induced gastric mucosal damage was evoked specifically only by the PMA injection or whether the ceramide pathway is also, in general, involved in the formation of gastric ulcers induced by various factors. It is also important to elucidate what kind of downstream molecules may be involved in gastric ulcer formation after ceramide activation.

Ceramide is produced from sphingosine (sphinganine) by sphingosine N-acyltransferase (ceramide synthase), which is potentially inhibited by fumonisin B1. Glucosylceramide synthase (GCS) is a ceramide glucosyl transferase that processes the sphingolipid ceramide [19]. This conversion of ceramide to glucosylceramide is prevented by *d,l,-threo*-1-phenyl-2-hexadecanoylamino-3-morpho-lino-1-propanol (PPMP) and N-butyldeoxynojirimycin (NB-DNJ) [20, 21]. The product, glucosylceramide, can be further elaborated with a variety of oligosaccharides to become glycosphingolipids called gangliosides such as GM3 (monosialoganglioside 3) and GD3 (disialoganglioside 3) [19] (Fig. 1).

To answer these questions, we investigated the ceramide formation and induction of apoptosis and gastric mucosal damage during the gastric ulcer formation process using a rat model of acetic acid-induced gastric ulcer, which is a representative experimental model of chronic gastric ulcer. We also examined the effects of two different kinds of glucosylceramide synthase inhibitors on the gastric ulcer formation induced by acetic acid, to investigate whether it was the ceramides themselves or their metabolites that were involved in the pathogenesis of the gastric ulcers.

Materials and Methods

Animals and Ulcer Induction

Male Sprague-Dawlay rats, weighing 200–250 g and maintained on standard laboratory chow (Oriental Yeast Mfg., Ltd., Tokyo, Japan) were used for all the experiments. All the animals were handled according to the guidelines of the Animal Research Committee of Keio University School of Medicine. The rats were denied any food for 24 h prior to the experiments, but were allowed access to tap water ad libitum. Gastric ulcers were induced by injection of an acetic acid solution [22]. Vehicle (water) was injected as a control. In brief, the abdomen of the animals, under anesthesia with 30 mg/kg of pentobarbital sodium, was opened via a midline incision. The stomach was exposed and 50 μl of either 20 % acetic acid or vehicle (water) was injected into the subserosa of the anterior wall of the glandular stomach using a microsyringe, followed by closure of the abdomen.

At different time intervals (24, 48, and 72 h) after the injection of acetic acid or vehicle, the rats were sacrificed with an overdose of sodium pentobarbital. Their stomachs were quickly removed, opened along the greater curvature, and rinsed with cold normal saline. The surface area of each lesion in the gastric mucosa was assessed visually by macroscopic examination. The ulcer area was calculated as an area of similarity ellipse (ulcer area = π**a***b**1/4; *a* major axis, *b* minor axis).

Administration of Various Inhibitors

To examine the changes in the gastric mucosal ceramide contents in this model, an inhibitor of sphingolipid biosynthesis, fumonisin B1 (FB1), was injected concomitantly (0.036–0.09 g/kg body weight) (Sigma) [23] with the acetic acid into the gastric subserosa. To determine the role of glucosylceramide in the acetic acid-induced ulcer formation, we used two types of inhibitors of glucosylceramide synthase, namely, *d,l,-threo*-1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol (PPMP) (0.0127–1.27 g/kg body weight) (Sigma-Aldrich) and N-butyldeoxynojirimycin (NB-DNJ) (0.11–11 g/kg body weight) (Sigma-Aldrich), which prevent the conversion of ceramide to glucosylceramide [20, 21]. These inhibitors were also injected concomitantly with acetic acid into the gastric subserosa. To prevent any systemic effects of the ceramide inhibitors as well as any

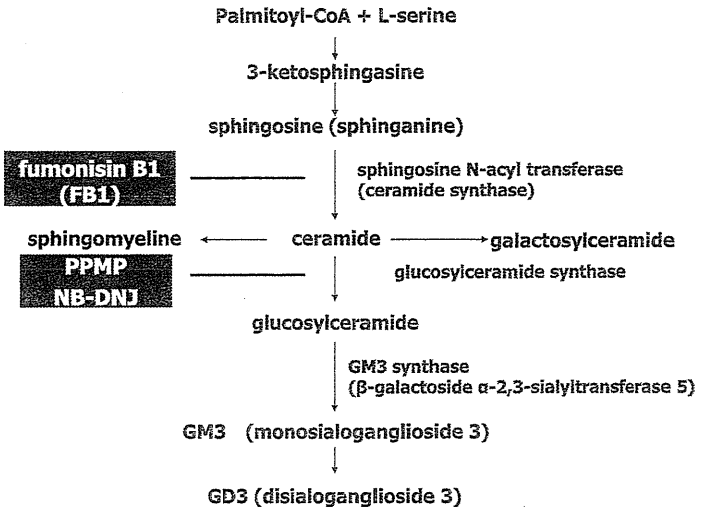

Fig. 1 Pathway of ceramide metabolism

possible interaction with acetic acid, ceramide inhibitors were injected locally with acetic acid by mixing just before the injection.

Determination of the Ceramide Contents in the Stomach

The time-course of changes of the ceramide contents in the stomach was examined. The excised stomachs were cut along the greater curvature and rinsed with physiological saline. Approximately 0.5 g of the tissue sample including the ulcer lesions was removed and minced, and lipid extraction was performed using a modified version of the method described by Bligh and Dyer [24]. After extracting the major lipids, the neutral lipids, including the ceramides, were separated by high-performance thin-layer chromatography (HPTLC) (Silicagel 60, Merck, Germany). The dried lipids were then resolved by thin-layer chromatography using petroleum ether/diethyl ether (7:3) as the first solvent, and chloroform/methanol (95:5) as the second solvent. After separating the lipids, the HPTLC plate was sprayed with a primulin reagent until it was thoroughly wet and then air-dried completely. The lipids were visualized under UV light at 365 nm and analyzed with a densitometer (Fluorchem^{TM} 8000, Alpha Innotech Co., San Leandro, CA, USA). Furthermore, the glucosylceramide and GM3 contents in the stomach were also examined according to the above-mentioned procedure. After the glycosphingolipids were separated by TLC, chloroform/methanol/0.2 % aqueous $CaCl_2$(60/35/8, by volume) was used as the developing agent for the TLC plates. GM3 was visualized by spraying the plate with orcinol-H_2SO_4 reagent. The lipids were visualized under UV light at 365 nm.

Determination of the Degree of Apoptosis in the Gastric Mucosa

Apoptosis was determined by immunohistochemical staining with a polyclonal antibody to ss-DNA. The area of the stomach containing the ulcer was rapidly excised and processed using routine techniques, followed by embedding in paraffin. Sections (4-μm thick) were then prepared and mounted on glass slides. Deparaffinized sections were treated with 3 % hydrogen peroxide for 20 min to block endogenous peroxide. Then, after blocking with 10 % non-immune serum for 10 min at room temperature, the sections were incubated for 40 min at room temperature with a primary antibody (anti-ss-DNA, polyclonal rabbit, DAKO, Carpinteria, CA, USA) diluted 1:100 with 0.1 % bovine serum albumin (BSA) in 0.05 M tris-buffered saline (TBS). The slides were washed three times with 0.05 M TBS-Tween for 5 min, followed by incubation for 30 min with rabbit peroxidase (DAKO). After washing for 5 min in TBS-Tween, the sections were stained using a diaminobenzidine reagent kit (Kirkegaard & Perry Laboratory Inc., Gaithersburg, USA) and observed under a microscope (Nikon ECLIPSE-E-600, Tokyo, Japan). Negative controls containing non-immune rabbit serum with omission of the primary antibody were also prepared. Staining for all antibodies was assessed in a blinded manner by the same observer.

Statistical Analysis

All results were expressed as the mean ± SEM. Differences among groups were evaluated using one-way analysis of variance (ANOVA) and Fisher's post hoc test. Statistical significance was set at $p < 0.05$.

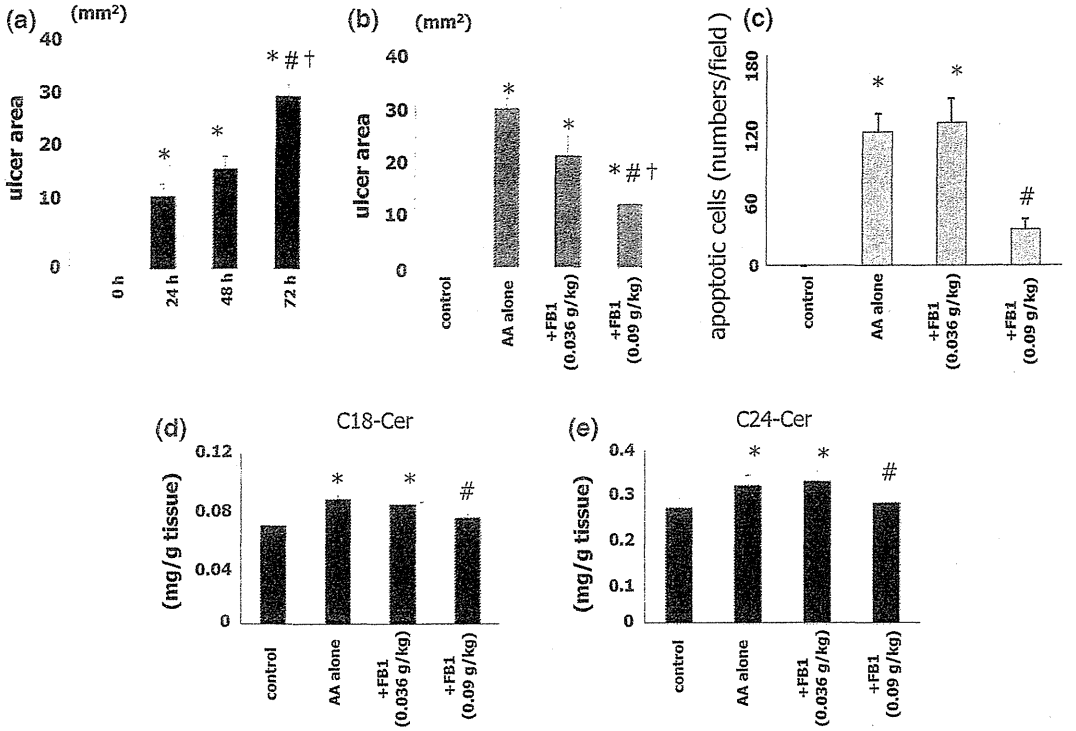

Fig. 2 **a** Time-course of changes in the area of the mucosal lesions after subserosal injection of 20 % acetic acid (50 µl). * $p < 0.05$ vs. 0 h, # $p < 0.05$ vs. 24 h, † $p < 0.05$ vs. 48 h. Each *bar* indicates mean value with SEM of six animals. **b** Inhibitory effects of different concentrations of fumonisin B1 (FB1; 0.036–0.09 g/kg body weight) on acetic acid-induced gastric ulcer formation at 72 h after the injection. Vehicle (water) was injected as a control. The ulcer index is expressed as the area of the mucosal lesions (mm^2). * $p < 0.05$ vs. control. # $p < 0.05$ vs. acetic acid alone. † $p < 0.01$ vs. FB1 (0.036 g/kg body weight). Values are mean ± SEM in six animals. **c** Effects of FB1 on the number of apoptotic cells appearing in the gastric mucosa at 72 h after acetic acid administration. The apoptotic cell number was determined in sections stained immunohistochemically with a polyclonal antibody against ss-DNA, and expressed as the average number of positively stained cells per microscopic field (×400). FB1 at 25 µM significantly attenuated the increase in the number of apoptotic cells induced by acetic acid administration. * $p < 0.05$ vs. control (vehicle); # $p < 0.05$ vs. acetic acid alone. Values are mean ± SEM in six animals. **d**, **e** Ceramide contents in the gastric mucosa at 72 h after acetic acid subserosal injection, and the inhibitory effect of FB1. Four samples were loaded on HPLC plates and densitometric analysis of the C18- (d) and C24- (e) ceramide contents was performed as described in "Materials and Methods". * $p < 0.05$ vs. control. # $p < 0.05$ vs. acetic acid alone. Values are mean ± SEM in six animals

Results

Effect of Fumonisin B1 on Acetic Acid-Induced Ulcer Formation

Figure 2a shows the time-course of changes in the area of the mucosal lesions, and Fig. 2b shows the inhibitory effects of different concentrations of fumonisin B1 (FB1; 0.036 and 0.09 g/kg body weight) on the area of the lesions after 72 h. The ulcers produced by the acetic acid injection began to form at the injection site in the stomach, expanded to their maximum size after 72 h, and healed gradually from day 5 to day 8 (data not shown). The ulcer formation was significantly inhibited by FB1 at the dose of 0.09 g/kg.

Figure 2c shows the number of apoptotic cells in the gastric mucosa at 72 h after the acetic acid injection as assessed immunohistochemically by light microscopy. A significant increase in the number of apoptotic cells was observed at 72 h after the acetic acid injection. FB1 at 0.036 g/kg did not significantly inhibit the acetic acid-induced apoptosis at 72 h, but the drug at 0.09 g/kg significantly attenuated the increase in the frequency of apoptosis induced by acetic acid at 72 h, which is consistent with the inhibition of ulcer formation by the drug. Figure 2d, e shows the C18- (2d) and C24- (2e) ceramide contents in the gastric mucosal lesions at 72 h after the acetic acid injection, and the inhibitory effect of FB1 on the accumulation of ceramides. The amounts of both the C18 and C24 ceramide were significantly increased at 72 h after the acetic acid injection, but not at 24 or 48 h after the injection (data not shown). The increase in the contents of the C18 and C24 ceramides in response to acetic acid injection was significantly attenuated by co-injection of FB1 (0.09 g/kg).

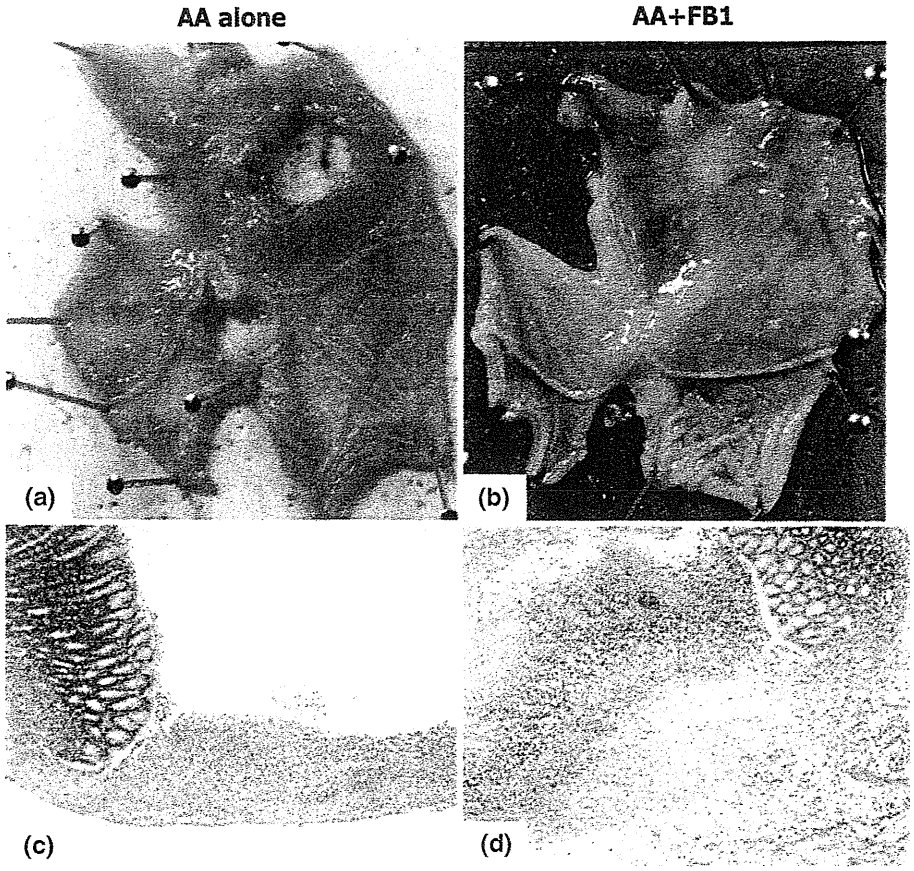

Fig. 3 Representative macroscopic findings were shown. **a** Acetic acid-induced gastric ulcer 72 h after subserosal injection. **b** Acetic acid-induced ulcer formation 72 h after subserosal injection was attenuated by the application of fumonisin B1 (0.09 g/kg body weight). **c**, **d** Representative hematoxylin-eosin (H&E)-stained histopathological findings were shown. **c** Acetic acid-induced ulcer 72 h after subserosal injection (×10). **d** Acetic acid-induced ulcer 72 h after subserosal injection was attenuated by fumonisin B1 (0.09 g/kg body weight) application (×10)

Figure 3a shows the representative macroscopic findings of acetic acid (AA)-induced gastric ulcer. As shown in Fig. 3b, AA-induced ulcer formation was attenuated by the application of FB1. Figure 3c shows the representative histopathological (hematoxylin-eosin staining) finding of AA-induced ulcer. As in Fig. 3d, such AA-induced-ulcer was attenuated by FB1 application.

Effect of PPMP on Acetic Acid-Induced Ulcer Formation

Figure 4a shows the effect of the glucosylceramide synthase inhibitor, PPMP, on acetic acid-induced gastric ulcer formation. PPMP at concentrations of over 0.127 g/kg body weight attenuated the sizes of the acetic acid-induced gastric mucosal lesions at 72 h after treatment.

Figure 4b shows the effect of PPMP on the number of apoptotic cells in the gastric mucosa at 72 h after acetic acid injection. Co-injection of PPMP with acetic acid at doses of over 0.127 g/kg significantly inhibited the acetic acid-induced increase in the number of apoptotic cells which is consistent with the inhibition of ulcer formation by the drug. Figure 4c, d shows a quantitative analysis of the contents of the C18- (4c) and C24- (4d) ceramide after acetic acid injection and the effect of PPMP. The increase in the amounts of both the C18- and C24-ceramide observed at 72 h after acetic acid injection was further enhanced by the concomitant injection of PPMP, and significantly greater amounts of the ceramides were found in the lesions following injection of PPMP at doses higher than 0.127 g/kg.

Effect of NB-DNJ on Acetic Acid-Induced Ulcer Formation

Figure 5a shows the effect of another glucosylceramide synthase inhibitor, NB-DNJ, on acetic acid-induced gastric ulcer formation. Co-injection of NB-DNJ with acetic acid at doses of over 1.1 g/kg body weight significantly attenuated the formation of the gastric mucosal lesions observed at 72 h after the acetic acid injection. Figure 5b shows the effect of NB-DNJ on the number of apoptotic cells appearing in the gastric mucosa at 72 h after the acetic acid injection. Co-injection of NB-DNJ at doses of over 0.11 g/kg body weight significantly attenuated the acetic acid-induced apoptosis in the gastric mucosa. Figure 5c, d shows the effect of NB-DNJ on the contents of the C18- (5c) and C24- (5d) ceramide at 72 h after acetic acid injection. The C18- and C24-ceramide contents significantly increased following

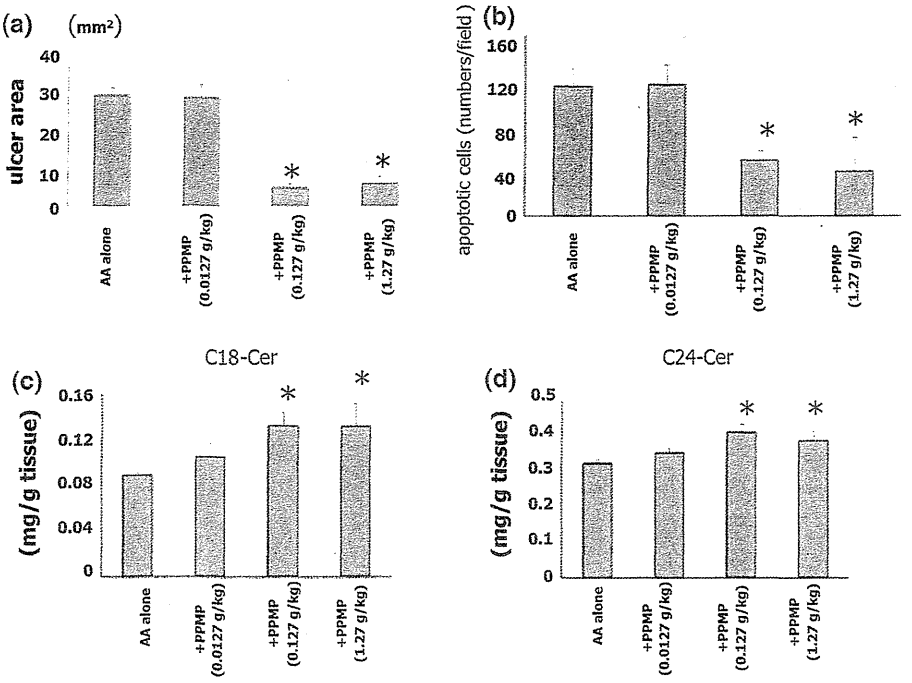

Fig. 4 Each *bar* indicates mean value with SEM of six animals. **a** Effect of the glucosylceramide synthase inhibitor, PPMP, on acetic acid-induced gastric ulcer formation at 72 h after treatment. PPMP (0.0127–1.27 g/kg body weight) was injected concomitantly with acetic acid into the gastric subserosa. * $p < 0.05$ vs. acetic acid alone. Values are mean ± SEM in six animals. **b** Effect of PPMP on the number of apoptotic cells appearing in the gastric mucosa at 72 h after acetic acid administration. PPMP (0.0127–1.27 g/kg body weight) was injected concomitantly with acetic acid into the gastric subserosa. * $p < 0.05$ vs. acetic acid alone. **c**, **d** C18- (c) and C24- (d) ceramide contents observed in the gastric mucosa at 72 h after acetic acid administration. PPMP (0.0127–1.27 g/kg body weight) was injected concomitantly with acetic acid into the gastric subserosa. * $p < 0.05$ vs. acetic acid alone. Values are mean ± SEM in six animals

co-injection of NB-DNJ (1.1 g/kg body weight) as compared with that observed following the injection of acetic acid alone, reflecting the decreased conversion of ceramide to glycosylceramide in these situations.

Glucosylceramide and GM3 on Acetic Acid-Induced Ulcer Formation

Figure 6 shows glucosylceramide and GM3 expressions in acetic acid-induced ulcer formation. The glucosylceramide levels were remarkably low in the acetic acid-induced ulcer group than in the control group. The level of ganglioside GM3 was observed to be high in the acetic acid-induced ulcer group. The expression of GM3 was suppressed and that of glucosylceramide were not restored by the treatment with glucosylceramide synthase inhibitors (PPMP, NB-DNJ).

Discussion

Our present results showing that the blockade of ceramide synthase by fumonisin B1 attenuates acetic acid-induced gastric ulcer formation suggest the importance of de novo ceramide synthesis in the process of ulcer formation induced by acetic acid. Although ceramides, which are derived from the hydrolysis of sphingomyelin in response to extracellular signals, appear to be important in most pathways [10], ceramide synthase-mediated processes, such as the acylation of sphinganine in the de novo biosynthetic pathway of sphingolipids as well as the reutilization of sphingosine derived from sphingolipid turnover [23, 25] may mainly account for the bioactive roles of ceramides in ulcer formation. In this study, we demonstrated an increase in the number of apoptotic cells in the gastric mucosa at 3 h after the injection of acetic acid, with subsequent extension of the lesion area containing apoptotic cells toward the submucosa, as well as a significant attenuation of the increase in acetic acid-induced apoptosis by co-injection of fumonisin B1. These findings suggest that the ceramide pathway may account for the acetic acid-induced ulcer formation via enhancing apoptotic cell death in the damaged mucosa. Our results also confirmed the significant role of the ceramide pathway in other specific experimental models of ulcers, such as PMA-induced gastric ulcers, besides that in the prototype model, namely, the model of acetic acid-induced ulcer [22].

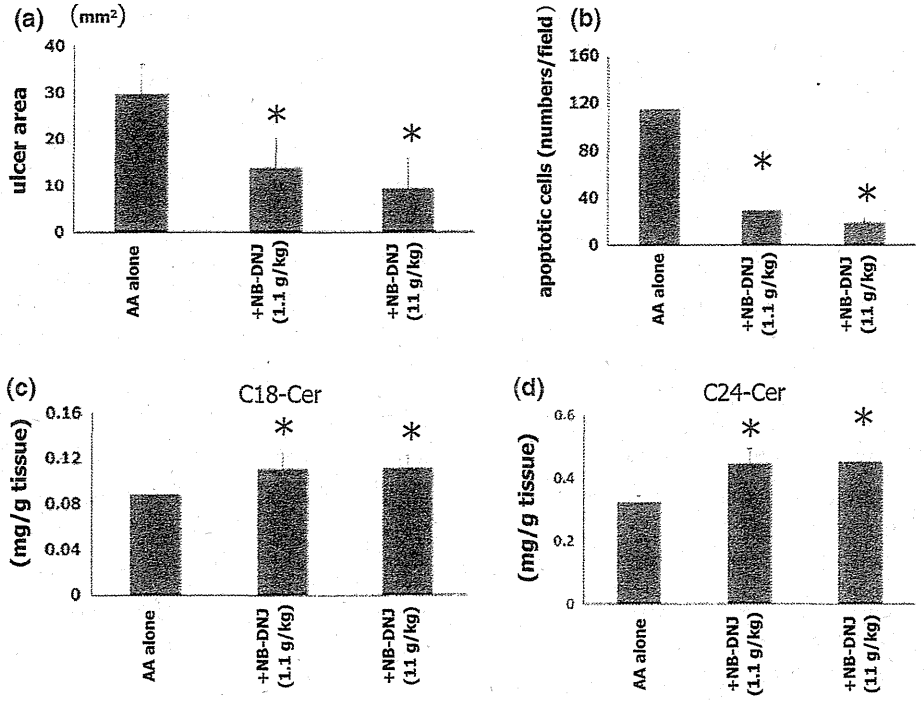

Fig. 5 Each *bar* indicates mean value with SEM of six animals. **a** Effect of NB-DNJ on acetic acid-induced gastric ulcer formation at 72 h after NB-DNJ (0.11–11 g/kg body weight) concomitant administration of the NB-DNJ with acetic acid into the gastric subserosa. * $p < 0.05$ vs. acetic acid alone. Values are the mean ± SEM in six animals. **b** Effect of NB-DNJ on the number of apoptotic cells appearing in the gastric mucosa at 72 h after acetic acid administration. NB-DNJ (0.11–11 g/kg body weight) was injected concomitantly with acetic acid into the gastric subserosa. * $p < 0.05$ vs. acetic acid alone. **c, d** Effect of NB-DNJ on the C18- (c) and C24- (d) ceramide contents observed in the gastric mucosa at 72 h after acetic acid administration. NB-DNJ (0.11–11 g/kg body weight) was injected concomitantly with acetic acid into the gastric subserosa. * $p < 0.05$ vs. acetic acid alone. Values are mean ± SEM in six animals

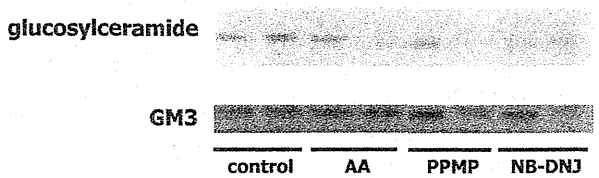

Fig. 6 Effect of PPMP (1.27 g/kg body weight) and NB-DNJ (11 g/kg body weight) on the glucosylceramide and GM3 after subserosal injection of 20 % acetic acid (50 µl). Representative pictures of thin layer chromatography of the glucosylceramide (*upper*) and GM3 (*lower*). Control: H_2O (sterile water) injection

In the present study, we demonstrated that the product of glucosylceramide synthase and not a ceramide itself induces apoptosis, and thus, glucosylceramide synthase inhibitors will decrease apoptosis. The reason for this decrease might be that apoptosis is induced by the product of glucosylceramide synthase.

In our previous manuscript [18], we only examined the increase in the ceramide (C18, C24) levels; we did not evaluate the levels of the ceramide metabolites. However, we showed that the increase in apoptosis in the gastric mucosa corresponded with the increase in the levels of C18 and C24 ceramide in the stomach wall, and that apoptosis was involved in the formation of gastric ulcers induced by PMA (phorbol 12-myristate 13-acetate). Although we previously suggested that ceramide or ceramide metabolites could be ulcerogenic [18], which of the two is ulcerogenic was not determined. In this context, in the previous study, a possibility that ceramide or ceramide metabolites could be a cause of ulcers was established. Our present results indicated that it is neither the C18- nor the C24-ceramide itself, but the respective metabolites that may be ulcerogenic, because we found, to our surprise, that glucosylceramide synthase inhibitors that reduce the degradation of ceramide can also attenuate the gastric mucosal damage induced by acetic acid. We used two types of inhibitors, namely, PPMP, a synthetic inhibitor of glucosylceramide synthase [26] and NB-DNJ, an N-alkylated imino sugar that blocks the activity of ceramide-specific glucosyltransferase which catalyzes the formation of glucosylceramide [27], both of which inhibit the conversion of ceramide to glucosylceramide. Indeed, the contents of the C18- and C24-ceramides were significantly augmented in the gastric mucosa when these inhibitors were injected

concomitantly with acetic acid. On the other hand, the acetic acid-induced tissue damage was attenuated with a decrease in the number of apoptotic cells under this condition, suggesting that inhibition of the synthesis of glucosylceramide, a precursor for neutral glycosphingolipids and gangliosides, effectively inhibits ulcer formation.

In the group of acetic acid-induced ulcer, while the glucosylceramide was not increased, the GM3 was remarkably accumulated as compared with the control group, suggesting that ceramide seems to be rapidly metabolized to ganglioside GM3 without accumulating intermediate metabolite such as glucosylceramide. On the other hand, in the group of acetic acid-induced ulcer treated with glucosylceramide synthesis inhibitors, the levels of both glucosylceramide and GM3 were not restored to the control level because glucosylceramide synthesis inhibitors could attenuate the pathway upstream of the glucosylceramide.

In the present study, we examined only the glucosylceramide pathway, and not the other pathways such as the sphingomyelin pathway. According to previous reports, GD3, a downstream metabolite of ceramide, is a key signaling intermediate leading to apoptosis [28], and the recently characterized trafficking of ganglioside GD3 to the mitochondria has revealed a novel function of this lipid as a death effector [29]; thus, the glucosylceramide pathway would be the main pathway, which is co-localized with the other pathways such as the sphingomyelin pathway.

Glucosylceramide synthase is a constitutively expressed type III integral membrane protein on the cytosolic side of the *cis/medial* Golgi membrane [30]. After its translocation to the Golgi lumen by an as yet undefined signaling mechanism, glucosylceramide is further metabolized to higher glycosphingolipids, including GM3 and GD3 gangliosides [11, 31]. It has been suggested previously that glycosylation of ceramide can protect cells from cancer drug-induced apoptosis. Accumulation of glucosylceramide was observed in multidrug-resistant tumor cells [32], and overexpression of glucosylceramide synthase in MCF-7 breast cancer cells conferred resistance to adriamycin and TNF-α [33]. These findings would support the idea that glycosylation of ceramide rather attenuates its capacity to act as a second messenger in apoptosis, although recently, it has been suggested that the natural ceramide species accumulating during the execution phase of apoptosis are not converted by glucosylceramide synthase to glucosylceramide, because this pool of ceramide is topologically segregated from glucosylceramide synthase [11]. In any event, the glucosylceramide formation *per se* does not appear to be a potentially toxic mediator in the acetic acid-induced gastric damage.

In addition to their role in the regulation of apoptosis, ceramides also provide the carbon backbone for the synthesis of complex glycosphingolipids within the Golgi network [34]. Inhibitors of glycosphingolipid biosynthesis have been used successfully as therapeutic agents for glycosphingolipid lysosomal storage diseases [35]. Healthy mice treated with NB-DNJ exhibited 70 % peripheral glycosphingolipid depletion [36], and clinical trials have shown the efficacy of these agents in patients with type 1 Gaucher's disease [37]. In addition, recently, ganglioside GD3 (GD3), a sialic acid-containing glycosphingolipid, has attracted considerable attention due to its emerging role as an effector of cell death by activating the mitochondrial-dependent apoptosis through sequential membrane permeability transition induction, cytochrome c release, and caspase activation [38]. De Maria et al. [39] showed that GD3 ganglioside mediates the propagation of CD95 (Fas)-generated apoptotic signals in hematopoietic cells, and that the pharmacological inhibition of GD3 synthesis and exposure to GD3 synthase antisense oligonucleotides prevented CD95-induced apoptosis. Another group recently demonstrated that the inhibition of glucosylceramide synthase, which blunted TNF-stimulated GD3 levels, abolished TNF-mediated apoptosis in human colon cancer cells [40], and also that *d-threo*-PDMP, an inhibitor of glucosylceramide synthase, blocked the TNF-α-induced translocation of GD3 to the mitochondria, thereby preserving its predominant localization at the cell surface in rat hepatocytes [41]. Since we previously demonstrated that an antibody against TNF-α significantly inhibited ulcer formation in the PMA-induced gastric ulcer model [17], TNF-α may also be involved in the process of acetic acid-induced ulcer formation. These previous reports suggesting that GD3 may play a significant role in TNF-α-mediated apoptosis are in close concordance with our present data indicating that inhibitors of glucosylceramide synthase successfully prevented the apoptosis and ulcer formation induced by acetic acid in spite of the significant accumulation of ceramide content observed in the gastric tissues.

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Conflict of interest None.

References

1. Konturek PC, Brzozowski T, Konturek SJ, et al. Apoptosis in gastric mucosa with stress-induced gastric ulcers. *J Physiol Pharmacol.* 1999;50:211–225.
2. Suzuki H, Ishii H. Role of apoptosis in *Helicobacter pylori*-associated gastric mucosal injury. *J Gastroenterol Hepatol.* 2000;15:D46–D54.

3. Suzuki H, Hibi T, Marshall BJ. *Helicobacter pylori*: present status and future prospects in Japan. *J Gastroenterol.* 2007;42: 1–15.
4. Suzuki H, Marshall BJ, Hibi T. Overview: *Helicobacter pylori* and extragastric disease. *Int J Hematol.* 2006;84:291–300.
5. Suzuki H, Iwasaki E, Hibi T. *Helicobacter pylori* and gastric cancer. *Gastric Cancer.* 2009;12:79–87.
6. Leung WK, To KF, Chan FK, Lee TL, Chung SC, Sung JJ. Interaction of *Helicobacter pylori* eradication and non-steroidal anti-inflammatory drugs on gastric epithelial apoptosis and proliferation: implications on ulcerogenesis. *Aliment Pharmacol Ther.* 2000;14:879–885.
7. Nagata H, Akiba Y, Suzuki H, Okano H, Hibi T. Expression of Musashi-1 in the rat stomach and changes during mucosal injury and restitution. *FEBS Lett.* 2006;580:27–33.
8. Liu ES, Cho CH. Relationship between ethanol-induced gastritis and gastric ulcer formation in rats. *Digestion.* 2000;62:232–239.
9. Hakomori S, Igarashi Y. Functional role of glycosphingolipids in cell recognition and signaling. *J Biochem (Tokyo).* 1995;118: 1091–1103.
10. Hannun YA. Functions of ceramide in coordinating cellular responses to stress. *Science.* 1996;274:1855–1859.
11. Tepper AD, Diks SH, van Blitterswijk WJ, Borst J. Glucosylceramide synthase does not attenuate the ceramide pool accumulating during apoptosis induced by CD95 or anti-cancer regimens. *J Biol Chem.* 2000;275:34810–34817.
12. Obeid LM, Linardic CM, Karolak LA, Hannun YA. Programmed cell death induced by ceramide. *Science.* 1993;259:1769–1771.
13. Dbaibo GS, Obeid LM, Hannun YA. Tumor necrosis factor-α (TNF-α) signal transduction through ceramide. Dissociation of growth inhibitory effects of TNF-α from activation of nuclear factor-kappa B. *J Biol Chem.* 1993;268:17762–17766.
14. Kolesnick R, Golde DW. The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signaling. *Cell.* 1994;77: 325–328.
15. Kim MY, Linardic C, Obeid L, Hannun Y. Identification of sphingomyelin turnover as an effector mechanism for the action of tumor necrosis factor α and γ-interferon. Specific role in cell differentiation. *J Biol Chem.* 1991;266:484–489.
16. Cifone MG, De Maria R, Roncaioli P, et al. Apoptotic signaling through CD95 (Fas/Apo-1) activates an acidic sphingomyelinase. *J Exp Med.* 1994;180:1547–1552.
17. Takeuchi T, Miura S, Wang L, et al. Nuclear factor-kappa B and TNF-α mediate gastric ulceration induced by phorbol myristate acetate. *Dig Dis Sci.* 2002;47:2070–2078.
18. Uehara K, Miura S, Takeuchi T, et al. Significant role of ceramide pathway in experimental gastric ulcer formation in rats. *J Pharmacol Exp Ther.* 2003;305:232–239.
19. Koltun E, Richards S, Chan V, et al. Discovery of a new class of glucosylceramide synthase inhibitors. *Bioorg Med Chem Lett.* 2011;21:6773–6777.
20. Shayman JA, Lee L, Abe A, Shu L. Inhibitors of glucosylceramide synthase. *Methods Enzymol.* 2000;311:373–387.
21. Platt FM, Neises GR, Karlsson GB, Dwek RA, Butters TD. N-butyldeoxygalactonojirimycin inhibits glycolipid biosynthesis but does not affect N-linked oligosaccharide processing. *J Biol Chem.* 1994;269:27108–27114.
22. Okabe S, Pfeiffer CJ. Chronicity of acetic acid ulcer in the rat stomach. *Am J Dig Dis.* 1972;17:619–629.
23. Wang E, Norred WP, Bacon CW, Riley RT, Merrill AH Jr. Inhibition of sphingolipid biosynthesis by fumonisins. Implications for diseases associated with Fusarium moniliforme. *J Biol Chem.* 1991;266:14486–14490.
24. Berg H, Magard M, Johansson G, Mathiasson L. Development of a supercritical fluid extraction method for determination of lipid classes and total fat in meats and its comparison with conventional methods. *J Chromatogr A.* 1997;785:345–352.
25. Merrill AH Jr, van Echten G, Wang E, Sandhoff K. Fumonisin B1 inhibits sphingosine (sphinganine) N-acyltransferase and de novo sphingolipid biosynthesis in cultured neurons in situ. *J Biol Chem.* 1993;268:27299–27306.
26. Maurer BJ, Melton L, Billups C, Cabot MC, Reynolds CP. Synergistic cytotoxicity in solid tumor cell lines between N-(4-hydroxyphenyl)retinamide and modulators of ceramide metabolism. *J Natl Cancer Inst.* 2000;92:1897–1909.
27. Svensson M, Frendeus B, Butters T, Platt F, Dwek R, Svanborg C. Glycolipid depletion in antimicrobial therapy. *Mol Microbiol.* 2003;47:453–461.
28. Lovat PE, Corazzari M, Di Sano F, Piacentini M, Redfern CP. The role of gangliosides in fenretinide-induced apoptosis of neuroblastoma. *Cancer Lett.* 2005;228:105–110.
29. Morales A, Colell A, Mari M, Garcia-Ruiz C, Fernandez-Checa JC. Glycosphingolipids and mitochondria: role in apoptosis and disease. *Glycoconj J.* 2004;20:579–588.
30. Jeckel D, Karrenbauer A, Burger KN, van Meer G, Wieland F. Glucosylceramide is synthesized at the cytosolic surface of various Golgi subfractions. *J Cell Biol.* 1992;117:259–267.
31. Ichikawa S, Hirabayashi Y. Glucosylceramide synthase and glycosphingolipid synthesis. *Trends Cell Biol.* 1998;8:198–202.
32. Lavie Y, Cao H, Bursten SL, Giuliano AE, Cabot MC. Accumulation of glucosylceramides in multidrug-resistant cancer cells. *J Biol Chem.* 1996;271:19530–19536.
33. Liu YY, Han TY, Giuliano AE, Ichikawa S, Hirabayashi Y, Cabot MC. Glycosylation of ceramide potentiates cellular resistance to tumor necrosis factor-α-induced apoptosis. *Exp Cell Res.* 1999;252:464–470.
34. Kolter T, Proia RL, Sandhoff K. Combinatorial ganglioside biosynthesis. *J Biol Chem.* 2002;277:25859–25862.
35. Platt FM, Neises GR, Reinkensmeier G, et al. Prevention of lysosomal storage in Tay-Sachs mice treated with N-butyldeoxynojirimycin. *Science.* 1997;276:428–431.
36. Platt FM, Reinkensmeier G, Dwek RA, Butters TD. Extensive glycosphingolipid depletion in the liver and lymphoid organs of mice treated with N-butyldeoxynojirimycin. *J Biol Chem.* 1997;272:19365–19372.
37. Cox T, Lachmann R, Hollak C, et al. Novel oral treatment of Gaucher's disease with N-butyldeoxynojirimycin (OGT 918) to decrease substrate biosynthesis. *Lancet.* 2000;355:1481–1485.
38. Garcia-Ruiz C, Colell A, Paris R, Fernandez-Checa JC. Direct interaction of GD3 ganglioside with mitochondria generates reactive oxygen species followed by mitochondrial permeability transition, cytochrome c release, and caspase activation. *FASEB J.* 2000;14:847–858.
39. De Maria R, Lenti L, Malisan F, et al. Requirement for GD3 ganglioside in CD95- and ceramide-induced apoptosis. *Science.* 1997;277:1652–1655.
40. Colell A, Morales A, Fernandez-Checa JC, Garcia-Ruiz C. Ceramide generated by acidic sphingomyelinase contributes to tumor necrosis factor-α-mediated apoptosis in human colon HT-29 cells through glycosphingolipids formation. Possible role of ganglioside GD3. *FEBS Lett.* 2002;526:135–141.
41. Garcia-Ruiz C, Colell A, Morales A, Calvo M, Enrich C, Fernandez-Checa JC. Trafficking of ganglioside GD3 to mitochondria by tumor necrosis factor-α. *J Biol Chem.* 2002;277: 36443–36448.

See related commentary on pg 868

ORIGINAL ARTICLE

Suppression of UV-Induced Wrinkle Formation by Induction of HSP70 Expression in Mice

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UV-induced wrinkle formation owing to the degeneration of the extracellular matrix (ECM) is a major dermatological problem in which abnormal activation of matrix metalloproteinases (MMPs) and elastases have important roles. Heat shock protein 70 (HSP70) has cytoprotective and anti-inflammatory activities. In this study, we examined the effect of HSP70 expression on UV-induced wrinkle formation. Mild heat treatment (exposure to heated water at 42 °C) of the dorsal skin of hairless mice induced the expression of HSP70. The long-term repeated exposure to UV induced epidermal hyperplasia, decreased skin elasticity, degeneration of ECM, and wrinkle formation, which could be suppressed in mice concomitantly subjected to this heat treatment. The UV-induced epidermal hyperplasia, decreased skin elasticity, and degeneration of ECM were less apparent in transgenic mice expressing HSP70 than in wild-type mice. UV-induced fibroblast cell death, infiltration of inflammatory cells, and activation of MMPs and elastase in the skin were also suppressed in the transgenic mice. This study provides evidence for an inhibitory effect of HSP70 on UV-induced wrinkle formation. The results suggest that this effect is mediated by various properties of HSP70, including its cytoprotective and anti-inflammatory activities. We propose that HSP70 inducers used in a clinical context could prove beneficial for the prevention of UV-induced wrinkle formation.

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INTRODUCTION

The skin is damaged by various environmental stressors, especially by long-term chronic solar UV radiation (photoaging). UV light can be separated according to wavelength, with UVB thought to have an important role in photoaging (Matsumura and Ananthaswamy, 2004; Rabe *et al.*, 2006). This process is mediated not only by direct UV-induced damage to the skin but also indirectly via the induction of inflammation and production of reactive oxygen species that are released from infiltrated leukocytes (Rabe *et al.*, 2006).

Both wrinkle formation and skin hyperpigmentation disorders are major dermatological problems. It is believed that epidermal hyperplasia owing to epidermal damage and inflammation, as well as alteration of the extracellular matrix (ECM) (such as damage to collagen fibers, suppression of collagen expression, disruption and degeneration of elastic fibers, and disruption of the epidermal basal membrane), has an important role in UV-induced wrinkle formation and decreased skin elasticity, which is closely linked to wrinkle formation (Talwar *et al.*, 1995; Imokawa, 2009; Rijken and Bruijnzeel, 2009). As these UV-induced phenomena can be reproduced to some extent in hairless mice exposed to long-term repeated exposure to UVB radiation, this animal model has been used to examine the mechanism of UV-induced wrinkle formation (Schwartz, 1988).

Matrix metalloproteinases (MMPs)-dependent qualitative and quantitative decreases in the ECM have an important role in UV-induced wrinkle formation. The activities of MMP-1, 2, 3, and 9 were increased by UVB irradiation in mouse and human skin (Inomata *et al.*, 2003; Rabe *et al.*, 2006), whereas the topical treatment of mouse skin with MMP inhibitors blocked UV-induced wrinkle formation, and decreased skin elasticity and basal membrane disruption (Inomata *et al.*, 2003). In mouse, MMP-2, 8, and 13, or MMP-2 and 9, are responsible for the degradation of collagen types I or IV, respectively, which constitute dermal collagen fibers or the epidermal basal membrane, respectively (Aimes and Quigley, 1995; Visse and Nagase, 2003; Kessenbrock *et al.*, 2010). In addition to collagenases (MMP-8 and 13), gelatinases (MMP-2 and 9) have important roles in UV-induced wrinkle formation, decreased skin elasticity, and basal membrane disruption (Inomata *et al.*, 2003). Tissue inhibitors of MMPs (TIMPs) also have important roles in these phenomena. The disruption

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Abbreviations: ECM, extracellular matrix; HSPs, heat shock proteins; MMPs, matrix metalloproteinases; TIMPs, tissue inhibitors of MMPs

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