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生体防御タンパク質に注目した、漢方薬の作用メカニズムの解明・有効成分の同定と新規治療薬の開発

平成 22 年度　総括研究報告書

主任研究者　水島　徹

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目次

I. 総括研究報告
生体防御タンパク質に注目した、漢方薬の作用メカニズムの解明・有効成分の同定と新規治療薬の開発 -----1

II. 研究成果の刊行に関する一覧表 -----8
III. 研究成果の刊行物・別刷 -----9

厚生労働科学研究費補助金（創薬基盤推進研究事業）
総括研究報告書

生体防御タンパク質に注目した、漢方薬の作用メカニズムの解明・有効成分の同定と新規治療薬の開発

主任研究者　水島　徹　熊本大学大学院医学薬学研究部教授

研究要旨

HSP70、及び SOD 誘導生薬のスクリーニングを行った。その結果、アルニカなど10種のHSP70誘導生薬、及びサルビアなど13種のSOD誘導生薬を発見した。これらのHSP70誘導生薬は、既存のHSP70誘導薬（テプレノン）よりも強力なHSP70誘導能、及び抗潰瘍作用（マウス）を示した。またサルビアは、PC-SODよりも強力な間質性肺炎、及び炎症性腸疾患抑制効果（マウス）を示した。

次に我々は、アルニカからHSP70誘導物質の単離、同定を試みた。オープンカラムで粗分けした後、分取用 HPLC で分画し、誘導物質の単離・構造決定に成功した。この誘導物質はテプレノンよりも強力なHSP70誘導能を有しており、現在アルツハイマー病抑制効果などを検討している。

一方サルビア等からのSOD誘導物質の単離、同定も進めている。サルビアに関しては、比活性を30倍以上上げることに成功したので、近い内に構造決定できると考えている。

A. 研究目的

我々はこれまで、様々な疾患に対して HSP や SOD などの生体防御タンパク質が保護的に働くことを報告してきた。HSP は様々なストレスによって誘導され、細胞をストレスに耐性化する。また我々は、HSP が抗炎症作用やタンパク質の変性を抑制する作用を持つことを発見した。一方我々はテプレノン（胃薬）が HSP を誘導する（但し、誘導能はあまり高くない）ことを発見し、テプレノンはこの作用により胃潰瘍を抑制していることを証明した。さらに小腸潰瘍や炎症性腸疾患（炎症と細胞死が主な原因）、及びアルツハイマー病などの神経変性疾患（タンパク質の変性が原因）に対しても HSP が保護的に働くこと、及びテプレノンが有効であることを見出した（現在これらの疾患に対するテプレノンの臨床試験を行っている）。

活性酸素による組織傷害は間質性肺炎や炎症性腸疾患（いずれも難病）などの炎症性疾患の主要な原因である。そこで活性酸素を消去する SOD は古くから注目されてきたが、その血中安定性が低いために医薬品としての開発は成功しなかった。そこで我々は、SOD にリン脂質を結合させ安定化させた PC-SOD を開発し、間質性肺炎、及び炎症性腸疾患に対する第二相臨床試験でその有効性を示した。しかし生物製剤である PC-SOD は生産コスト、及び製剤としての安定性に問題があり、低分子の SOD 誘導薬が望まれている。

長年使われてきた漢方薬は、その安全性・有効性が確認されていることから、医薬品原料として注目されてきた。しかし現在まで、漢方薬由来の物質が新規医薬品として認可されたケースは少ない。我々はその原因として、そのような医薬品開発の多くが、受容体や酵素の阻害など西洋医薬品と同じ機構をターゲットとしており、漢方薬の特徴である緩やかな作用・副作用の少なさとマッチしていないこと、即ち漢方薬は西洋医薬品とは違う独自のターゲット（生体防御タンパク質の効果を高めるなど）を持っていることを考えている（研究計画で述べるように、この考えを支持する成果を最近あげた）。そこで本研究で我々は、漢方薬（生薬）ライブラリーから HSP 誘導生薬、及び SOD 誘導生薬を検索し、誘導物質の同定、及び動物モデルでの評価を行い、疾患治療薬として開発する化合物を決定する。

ゲノム創薬などにより、21 世紀は新薬の開発ラッシュになると予想されていた。しかし現実には、発売される新薬の数は年々減少しており、製薬企業は医薬品開発戦略の変更を迫られている。この主な原因は臨床試験で発生する副作用であり、作用の強い医薬品より副作用の少ない医薬品を開発すべきであると考えられる。これまでの医薬品は受容体や酵素の阻害・活性化剤が主であり、生体内のバランスを大きく変えることにより副作用を導くと考えられる。そこで我々は、疾患というストレスに対して生体が自らを守るために誘導する生体防御タンパク質を増強させるタイプの医薬品が有用であると考えている。即ち、疾患に対する生体防御タンパク質の誘導が不十分であるために疾患が発症すると考え、医薬品によりその不足分を補うという考えである。生体が本来持っている反応を助けるだけであるので、副作用を起こしにくいと期待される。HSP 誘導薬や PC-SOD が間質性肺炎などの難病に有効であるという臨床結果は、このような医薬品は安全面で優れているだけでなく、従来型の医薬品では効果をあげられなかった疾患にも有効であることを示唆している。

本研究が成功すれば、種々の難病に対する治療薬が生まれるだけでなく、新しい医薬品開発戦略（生体防御タンパク質をターゲットとする医薬品を検索する材料として漢方薬を用いる）を製薬企業へ示すことになり、大きな波及効果が期待できる。

B. 研究方法

最近我々は、共同研究している中国企業（北京泰徳製薬）から得た漢方薬（生薬）ライブラリー（約 600 種）から HSP の誘導生薬をスクリーニングし、テプレノンよりも強力、かつ安全な数多くの HSP 誘導生薬を得た（特許出願済み）。我々はこの中からヤバツイを選択し、その HSP 誘導物質の同定に成功した（特許出願準備中）。この誘導物質

を小腸潰瘍、炎症性腸疾患、アルツハイマー病の動物モデルで評価したところ、テプレノンよりも強力な効果を示した。この結果は、漢方薬（生薬）ライブラリーからスクリーニングした HSP 誘導物質が医薬品として有用であることを示唆している。また最近我々は、HSP が間質性肺炎（有効な治療薬はなく、致死率は 80%を超える）、COPD（世界中で患者数が増大しており、有効な治療薬がない）、及び ALS やハンチントン舞踏症などの神経変性疾患の発症を抑制することを見出した。

そこで本研究で我々は、この漢方薬（生薬）ライブラリーをさらに充実させ、HSP 誘導生薬のスクリーニングを行い、有望な生薬を複数選択する。そして、誘導物質の同定、及び動物モデルでの評価を行い、種々の疾患治療薬として開発する HSP 誘導物質を決定する。

一方最近我々は、PC-SOD が間質性肺炎や炎症性腸疾患だけでなく、活性酸素による組織傷害がその主な原因となっている、腎炎、肝炎、膵炎、喘息、COPD、アトピー性皮膚炎の動物モデルにおいて有効性を示すことを見出した。そこで本研究で我々は、上述のライブラリーから SOD 誘導物質を検索・同定し、種々の疾患治療薬として開発する SOD 誘導物質を決定する。

（1）　漢方薬（生薬）ライブラリーの整備

上述の HSP 誘導生薬（ヤバツイ）は、化粧品として商品化が決定している。この成果を評価した北京泰徳製薬は中国政府から特別の許可を得て、2000 種以上の生薬を供与してくれることになった（最近では生薬を海外に出すことに中国政府は慎重になっており、このようなライブラリーを有する研究機関は国内にほとんどない）。そこでこの生薬の溶解法や投与法を確立し、スクリーニングの準備を行う。

（2）　HSP、及び SOD 誘導生薬のスクリーニングと、誘導物質の同定

HSP、あるいは SOD 遺伝子プロモーターの下流にルシフェラーゼ遺伝子を挿入したプラスミドを導入した細胞を用いて一次スクリーニングを行い、イムノブロット法で二次スクリーニングを行う。毒性の少ない誘導薬を得たいので、三次スクリーニングではその生薬の細胞毒性を調べ、細胞毒性を示さない濃度で HSP、あるいは SOD を誘導するものを選択する。四次スクリーニングではその生薬をマウスに投与し、目的のタンパク質を誘導するかを検討する。

これらの結果から有望な生薬を選択し、その誘導物質の同定を行う。オープンカラムで粗分けした後、分取用 HPLC で分画し、誘導物質の構造を決定する。合成可能な物は合成し、難しいものは大量の生薬から精製する。

（3）　HSP、及び SOD 誘導物質の疾患治療薬としての評価

それぞれの誘導物質の効果をまず試験管内で評価する。HSP 誘導物質に関しては、炎症抑制作用、細胞保護作用、及びタンパク質凝集抑制作用の程度を調べる。また SOD 誘導物質に関しては、活性酸素消去作用を調べる。次にその効果が HSP、あるいは SOD を介しているかを、siRNA を用いて検証する。

最終的には、種々の疾患動物モデルを用いて評価する。治療効果が見られた場合、その効果が HSP、あるいは SOD を介しているかを、そのタンパク質を誘導出来ないマウスを用いて判断する。有用な薬理効果が見られた場合には、他の臓器の状態を精査し副作用が表れていないかを調べる。尚、HSP 誘導物質の場合は GGA と、SOD 誘導物質の場合は PC-SOD と治療効果を比較する。結果を総合的に判断し、それぞれの疾患治療薬として開発する誘導物質を決定する。

C. 研究結果

本年一月我々は、自ら発見した HSP 誘導生薬（ヤバツイ）を化粧品として発売した。世界初の HSP 誘導化粧品として評価され、この分野のトップ商品になっている。この成果を評価した北京泰徳製薬は、中国政府から特別の許可を得て、2000 種以上の生薬を我々に提供してくれた。我々はこれら生薬の溶解法や投与法を確立し、2500 種以上の生薬からなる生薬ライブラリーを確立した（最近では生薬を海外に出すことに中国政府は慎重になっており、このようなライブラリーを有する研究機関は国内にほとんどない）。

そしてこのライブラリーを用いて、HSP70、及び SOD 誘導生薬のスクリーニングを行った。その結果、アルニカなど 10 種の HSP70

誘導生薬、及びサルビアなど 13 種の SOD 誘導生薬を発見した。これらの HSP70 誘導生薬は、既存の HSP70 誘導薬（テプレノン）よりも強力な HSP70 誘導能、及び抗潰瘍作用（マウス）を示した。またサルビアは、PC-SOD よりも強力な間質性肺炎、及び炎症性腸疾患抑制効果（マウス）を示した。尚、アルニカは来年度発売する化粧品に配合されることが決定され、我々はアルニカの特許を化粧品会社へライセンスアウトし、現在共同で化粧品開発を行っている。

次に我々は、アルニカから HSP70 誘導物質の単離、同定を試みた。オープンカラムで粗分けした後、分取用 HPLC で分画し、誘導物質の単離・構造決定に成功した。この誘導物質はテプレノンよりも強力な HSP70 誘導能を有しており、現在アルツハイマー病抑制効果などを検討している。

一方サルビア等からの SOD 誘導物質の単離、同定も進めている。サルビアに関しては、比活性を 30 倍以上上げることに成功したので、近い内に構造決定できると考えている。

このように生体防御タンパク質誘導生薬のスクリーニングがうまくいっているので、研究計画を発展的に変更し、HSP47、及び HO-1（我々の研究から、医薬品や化粧品のターゲット分子として有望であることが示唆されている生体防御タンパク質）の誘導生薬のスクリーニングも開始することにした（研究計画・方法参照）。

D.考察

結果の欄に記載した

E.結論

このように平成 22 年度の我々の研究により、数多くの有望な生薬が発見された。

F.健康危険情報

該当なし

G.研究発表

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28　水島徹　既存薬の新しい薬理作用の発見と、適応拡大への応用　鹿児島県薬剤師会特別講演　(2011)（鹿児島）

29　水島徹　皮膚における熱ショック蛋白質の役割とその応用　第3回熊本乾癬病診連携フォーラム　(2011)（熊本）

30　水島徹　トランスクリプトソーム解析による医薬品の副作用機構の解明と、その副作用感受性診断、及び創薬への応用　創薬バイオマーカー探索研究事業研究発表会での招待講演　(2011)（東京）

31　水島徹　既存薬の作用分子機構の解明と創薬への展開　日本薬学会での受賞講演　(2011)（東京）

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

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Yamakawa, N., Suemasu, S., Kimoto, A., Arai, Y., Ishihara, T., Yokomizo, K., Okamoto, Y., Ohtsuka, M., Tanaka, K. and Mizushima, T.	Low direct cytotoxicity of loxoprofen on gastric mucosal cells.	***Biol. Pharm. Bull.***	33	398-403.	2010
Matsuda, M., Hoshino, T., Yamashita, Y., Tanaka, K., Maji, D., Sato, K., Adachi, H., Sobue, G., Ihn, H., Funasaka, Y. and Mizushima, T.	Prevention of ultraviolet B radiation-induced epidermal damage by expression of heat shock protein 70.	***J. Biol. Chem.***	285	5848-5858.	2010
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Low Direct Cytotoxicity of Loxoprofen on Gastric Mucosal Cells

Naoki YAMAKAWA, Shintaro SUEMASU, Ayumi KIMOTO, Yasuhiro ARAI, Tomoaki ISHIHARA, Kazumi YOKOMIZO, Yoshinari OKAMOTO, Masami OTSUKA, Ken-ichiro TANAKA, and Tohru MIZUSHIMA*

Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University; Kumamoto 862–0973, Japan.
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Pro-drugs of non-steroidal anti-inflammatory drugs (NSAIDs), such as loxoprofen are widely used for clinical purposes because they are not so harmful to the gastrointestinal mucosa. We recently showed that NSAIDs such as indomethacin and celecoxib have direct cytotoxicity (ability to induce necrosis and apoptosis in gastric mucosal cells) due to their membrane permeabilizing activities, which is involved in NSAID-induced gastric lesions. We show here that under conditions where indomethacin and celecoxib clearly induce necrosis and apoptosis, loxoprofen and its active metabolite loxoprofen-OH, do not have such effects in primary culture of guinea pig gastric mucosal cells. Loxoprofen and loxoprofen-OH induced apoptosis more effectively in cultured human gastric cancer cells than in the primary culture. Loxoprofen and loxoprofen-OH exhibited much lower membrane permeabilizing activities than did indomethacin and celecoxib. We thus consider that the low direct cytotoxicity of loxoprofen observed *in vitro* is involved in its relative safety on production of gastric lesions in clinical situation.

Key words loxoprofen; gastric mucosal cell; membrane permeabilization; gastric lesion

Non-steroidal anti-inflammatory drugs (NSAIDs), such as indomethacin, are a useful family of therapeutics.[1] An inhibitory effect of NSAIDs on cyclooxygenase (COX) activity is responsible for their anti-inflammatory actions because COX is an enzyme essential for the synthesis of prostaglandins (PGs), such as PGE_2, which have a strong capacity to induce inflammation. On the other hand, NSAID use is associated with gastrointestinal complications.[2—4]

In 1991, two subtypes of COX, COX-1 and COX-2, which are responsible for the majority of COX activity at the gastrointestinal mucosa and in tissues with inflammation, respectively, were identified.[5,6] Since PGE_2 has a strong protective effect on the gastrointestinal mucosa, it is reasonable to speculate that selective COX-2 inhibitors maintain anti-inflammatory activity without gastrointestinal side-effects. In fact, a greatly reduced incidence of gastroduodenal lesions has been reported for selective COX-2 inhibitors (such as celecoxib and rofecoxib).[7—9] However, a recently raised issue concerning the use of selective COX-2 inhibitors is their potential risk for cardiovascular thrombotic events.[10,11] This may be due to the fact that prostacyclin, a potent anti-aggregator of platelets and a vasodilator, is mainly produced by COX-2 in vascular endothelial cells, while thromboxane A_2, a potent aggregator of platelets and a vasoconstrictor, is mainly produced by COX-1 in platelets.[12—14] Because of this concern, rofecoxib was withdrawn from the worldwide market. Therefore, NSAIDs exhibiting gastrointestinal safety, other than selective COX-2 inhibitors, are clinically important.

The inhibition of COX by NSAIDs is not the sole explanation for the gastrointestinal side-effects of NSAIDs.[15] We have recently demonstrated that NSAIDs induce necrosis and apoptosis in cultured gastric mucosal cells and at gastric mucosa in a manner independent of COX inhibition.[16—20] We clearly showed that the primary target of NSAIDs for induction of necrosis and apoptosis is cytoplasmic membranes.[16,18] As for the molecular mechanism governing this apoptosis, we have proposed the following pathway. Permeabilization of cytoplasmic membranes by NSAIDs stimulates Ca^{2+} influx and increases intracellular Ca^{2+} levels, which in turn induces the endoplasmic reticulum (ER) stress response.[16,21,22] In this response, an apoptosis-inducing transcription factor, CCAAT/enhancer-binding protein (C/EBP) homologous transcription factor (CHOP), is induced and CHOP induces expression of p53 up-regulated modulator of apoptosis (PUMA) and resulting translocation and activation of Bax, mitochondrial dysfunction, activation of caspases and apoptosis.[17,23] Furthermore, we have suggested that both COX inhibition and gastric mucosal cell death are required for the formation of NSAID-induced gastric lesions *in vivo*.[20,24]

Loxoprofen has been used clinically for a long time as a standard NSAID in Japan, and clinical studies have suggested that it is safer than other NSAIDs, such as indomethacin.[25,26] Loxoprofen is a pro-drug, which is converted (by reduction of the cyclopentanone moiety) to its active metabolite (the *trans*-alcohol metabolite of loxoprofen, loxoprofen-OH) by aromatic aldehyde-ketone reductase only after absorption by the gastrointestinal tract.[27] However, the direct cytotoxicity and membrane permeabilization activity of loxoprofen has not been tested. In this study, we found that loxoprofen and loxoprofen-OH have relatively lower membrane permeabilization activities and cytotoxic effects on gastric mucosal cells than other NSAIDs. Based on these observations, we consider that the low direct cytotoxicity of loxoprofen will render its use clinical safe on the gastrointestinal mucosa.

MATERIALS AND METHODS

Chemicals and Media RPMI 1640 was obtained from Nissui Pharmaceutical Co. Fetal bovine serum (FBS) and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were from Sigma Co. Loxoprofen and loxoprofen-OH were kindly gifted from Daiichi-Sankyo Co. Indomethacin was from Wako Co. Celecoxib was from LKT Laboratories Inc.

* To whom correspondence should be addressed. e-mail: mizu@gpo.kumamoto-u.ac.jp
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Egg phosphatidylcholine (PC) was from Kanto Chemicals Co. Male guinea pigs weighing 200—300 g were purchased from Kyudo Co. The experiments and procedures described here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institute of Health and were approved by the Animal Care Committee of Kumamoto University.

***In Vitro* Assay of Cytotoxicity of NSAIDs and COX-Inhibition** Gastric mucosal cells were isolated from guinea pig fundic glands as described previously.[28,29] Isolated gastric mucosal cells were cultured for 12 h in RPMI 1640 containing 0.3% v/v FBS, 100 U/ml ampicillin and 100 μg/ml streptomycin in type-I collagen-coated plastic culture plates under the conditions of 5% CO_2/95% air and 37 °C. After removing non-adherent cells, cells attached to the plate were used. Guinea pig gastric mucosal cells prepared under these conditions were previously characterized, with the majority (about 90%) of cells being identified as pit cells.[28,30] Human gastric adenocarcinoma (AGS) cells were cultured on plastic culture plates without collagen-coating under the same conditions.

NSAIDs were dissolved in dimethyl sulfoxide (DMSO). Cells were exposed to NSAIDs by changing the entire bathing medium.

We used MTT assay for monitoring cell viability. Cells were incubated for 2 h with MTT solution at a final concentration of 0.5 mg/ml. Isopropanol and hydrochloric acid were added to the culture medium at final concentrations of 50% and 20 mM, respectively. The optical density of each sample at 570 nm was determined spectrophotometrically using a reference wavelength of 630 nm.[31]

The amount of PGE_2 in the medium was determined using an EIA kit (Cayman, Ann Arbor, MI, U.S.A.) according to the manufacturer's protocol.

Apoptotic DNA fragmentation was monitored as previously described.[31] Cells were collected using a rubber policeman and suspended in 20 μl of lysis buffer, consisting of 50 mM Tris–HCl (pH 7.8), 10 mM ethylenediaminetetraacetic acid (EDTA), and 0.5% sodium-*N*-lauroylsarcosinate. Proteinase K was added to a final concentration of 1 mg/ml, and the lysate was incubated at 50 °C for 2 h. RNaseA was then added to a final concentration of 0.5 mg/ml and incubated at 50 °C for 30 min. These samples were analyzed by 2% agarose gel electrophoresis in the presence of 0.5 μg/ml ethidium bromide.

Apoptotic chromatin condensation was monitored as described previously.[31] Cells were washed with PBS, stained with 10 μg/ml Ho 342 and observed under a fluorescence microscope.

Membrane Permeability Assay Membrane permeability assays were performed as described previously.[16,18,32] Liposomes were prepared using the reversed-phase evaporation method. Egg PC (10 μmol, 7.7 mg) was dissolved in chloroform/methanol (1 : 2, v/v), dried, and dissolved in 1.5 ml of diethyl ether. This was followed by the addition of 1 ml of 100 mM calcein-NaOH (pH 7.4). The mixture was sonicated to obtain a homogenous emulsion. The diethyl ether solvent was removed using a conventional rotary evaporator under reduced pressure at 25 °C. The resulting suspension of liposome was centrifuged and washed twice with fresh buffer A (10 mM phosphate buffer, containing 150 mM NaCl) to remove untrapped calcein. The final liposome precipitate was re-suspended in 5 ml buffer A. A 0.3 ml aliquot of this suspension was diluted with 19.7 ml of buffer A, following which 400 μl of this suspension was incubated at 30 °C for 10 min in the presence of the NSAID under investigation. The release of calcein from liposomes (the amount of calcein outside the liposomes) was determined by measuring fluorescence intensity at 520 nm (excitation at 490 nm), because the calcein fluoresces very weakly when at high concentrations (when calcein is trapped in liposomes) due to self-quenching.

Hemolysis in erythrocytes were monitored as described[33,34] with some modifications. Rat erythrocytes were washed twice with buffer A (5 mM HEPES/NaOH (pH 7.4) and 150 mM NaCl) and then suspended in fresh buffer A at a final concentration of 0.5% hematocrit (5×10^7 cells/ml). After incubation with NSAIDs for 10 min at 30 °C, hemolysis was estimated by measuring the absorbance at 520 nm.

Statistical Analyses All values are expressed as the mean$\pm$S.E.M. The Tukey test or the Student's *t*-test for unpaired results was used to evaluate differences between more than three groups or between two groups, respectively.

RESULTS AND DISCUSSION

Necrosis- and Apoptosis-Inducing Activities of Loxoprofen and Loxoprofen-OH in Primary Culture of Gastric Mucosal Cells We previously reported that NSAIDs induce either necrosis or apoptosis depending on treatment conditions; short-term (1 h) treatment of primary cultures of guinea pig gastric mucosal cells with relatively high concentrations of NSAIDs (2.5 mM for indomethacin and 0.2 mM for celecoxib) and long-term (16—24 h) treatment of these cells with relatively low concentrations of NSAIDs (1 mM for indomethacin and 0.05 mM for celecoxib) induces necrosis and apoptosis, respectively.[18,19,24] Loxoprofen and loxoprofen-OH were tested here for their ability to induce necrosis and apoptosis. Consistent with previous reports,[18,19,24] cell viability decreased in a dose-dependent manner when guinea pig gastric mucosal cells in primary culture were treated with indomethacin or celecoxib for 1 h. In contrast, loxoprofen and loxoprofen-OH decreased cell viability to a much lesser extent under the same experimental conditions; cell viability of more than 60% was observed even with the highest concentration (20 mM) of loxoprofen and loxoprofen-OH (Fig. 1). We confirmed that cell death highlighted in Fig. 1 was medi-

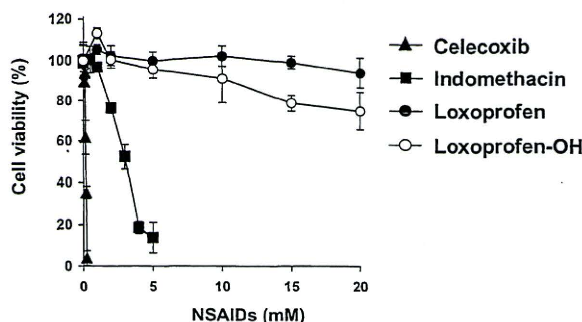

Fig. 1. Necrosis Induced by NSAIDs in Primary Culture of Gastric Mucosal Cells

Cultured guinea pig gastric mucosal cells were incubated with indicated concentrations of NSAIDs for 1 h. Cell viability was determined by the MTT method. Values are mean$\pm$S.E.M. (n=3).

ated by necrosis given that no accompanying apoptotic DNA fragmentation or apoptotic chromatin condensation were evident (data not shown).

Similar results to the above were obtained when apoptosis was induced. Treatment of cells for 18 h with indomethacin or celecoxib decreased cell viability in a dose-dependent manner (Fig. 2A), which is also consistent with previous reports.[18,19,24] Loxoprofen and loxoprofen-OH showed very low activities for decreasing cell viability under these conditions (Fig. 2A). Because cell death as highlighted in Fig. 2 was accompanied by apoptotic DNA fragmentation and apoptotic chromatin condensation (Figs. 2B, C), it is most likely to have been mediated by apoptosis. Overall, the results in Figs. 1 and 2 show that loxoprofen and loxoprofen-

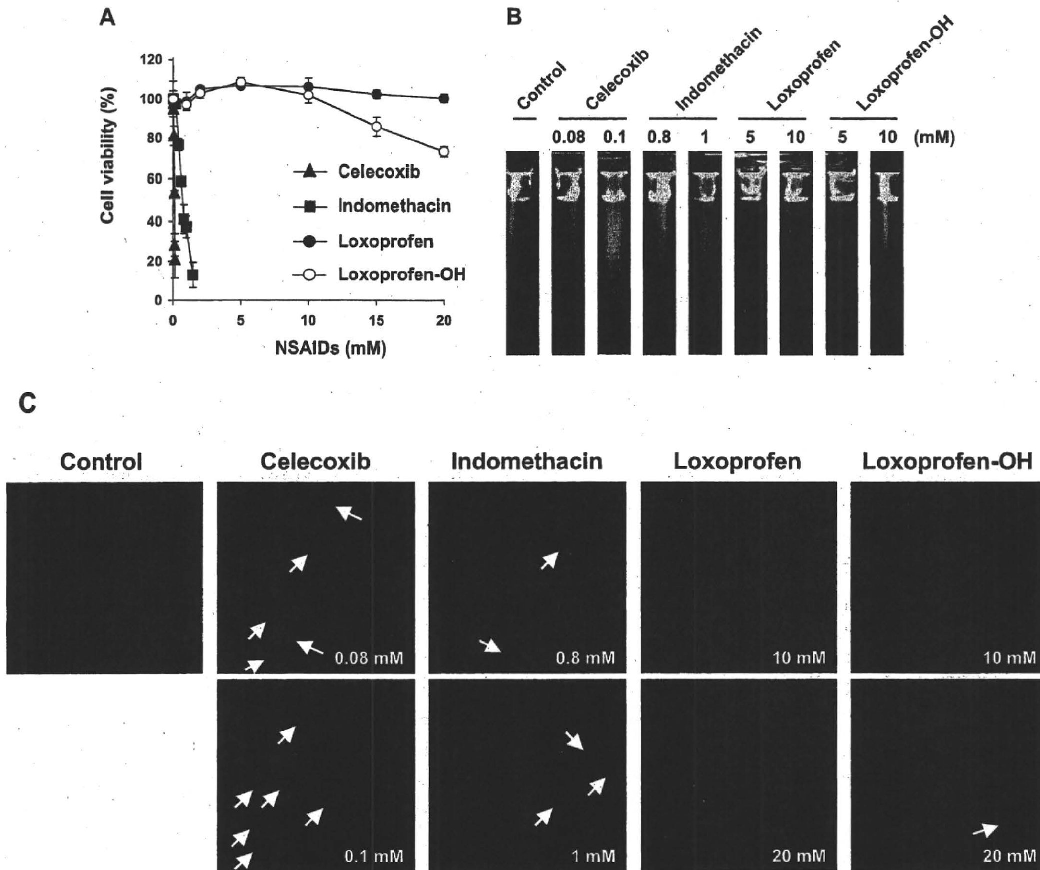

Fig. 2. Apoptosis Induced by NSAIDs in Primary Culture of Gastric Mucosal Cells

Cultured guinea pig gastric mucosal cells were incubated with indicated concentrations of NSAIDs for 18 h. Cell viability was determined by the MTT method. Values are mean±S.E.M. ($n=3$) (A). Apoptotic DNA fragmentation (B) and chromatin condensation (C) were monitored as described in Materials and Methods.

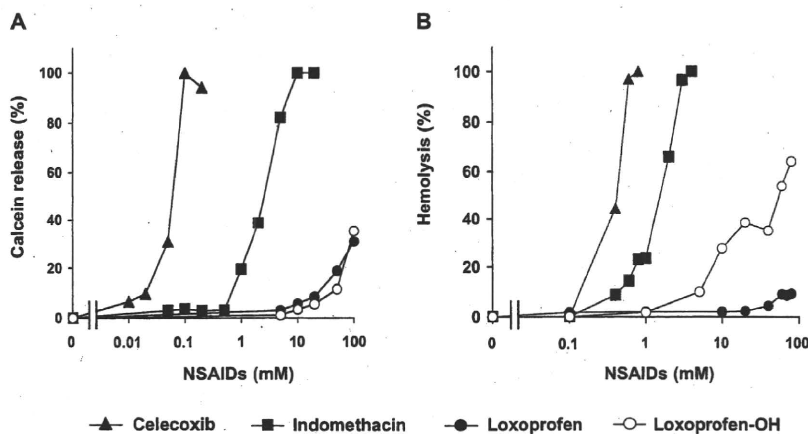

Fig. 3. Membrane Permeabilization by NSAIDs

Calcein-loaded liposomes were incubated for 10 min at 30 °C with indicated concentrations of NSAIDs. The release of calcein from liposomes was determined by measuring fluorescence intensity. Melittin (10 μM) was used to determine the 100% level of membrane permeabilization (A). Rat erythrocytes were incubated in the presence of each of NSAIDs for 10 min at 30 °C. Hemolysis was estimated by measuring the absorbance at 520 nm (B).

OH induce necrosis and apoptosis to a lesser extent than do indomethacin and celecoxib. Furthermore, although the metabolic conversion of loxoprofen to loxoprofen-OH drastically increases the inhibitory activity on COX, this conversion does not seem to be so apparently associated with a similar increase in direct cytotoxicity.

Membrane Permeabilization Activities of Loxoprofen and Loxoprofen-OH The ability of loxoprofen and loxoprofen-OH to permeabilize the membranes of calcein-loaded liposomes was examined. Calcein fluoresces very weakly when at high concentrations due to self-quenching. Thus, the addition of membrane permeabilizing drugs to a medium containing calcein-loaded liposomes should cause an increase in fluorescence by releasing calcein trapped within the liposomes.[18] As shown in Fig. 3A, indomethacin and celecoxib increased the calcein fluorescence in a dose-dependent manner, which is consistent with previous findings.[18] Loxoprofen and loxoprofen-OH also increased the calcein fluorescence, suggesting that they caused membrane permeabilization; however, as the concentrations of loxoprofen and loxoprofen-OH required for membrane permeabilization were much higher than those of indomethacin and celecoxib, their abilities to permeabilize membranes were thus very weak.

Measurement of hemolysis is a standard method for testing the membrane permeabilization activities of drugs. As shown in Fig. 3B, all of the tested NSAIDs caused hemolysis of erythrocytes. The relative potency of each NSAID for hemolysis was approximately similar to that for permeabilization of calcein-loaded liposomes. Celecoxib showed the most potent activity for hemolysis, followed by indomethacin and

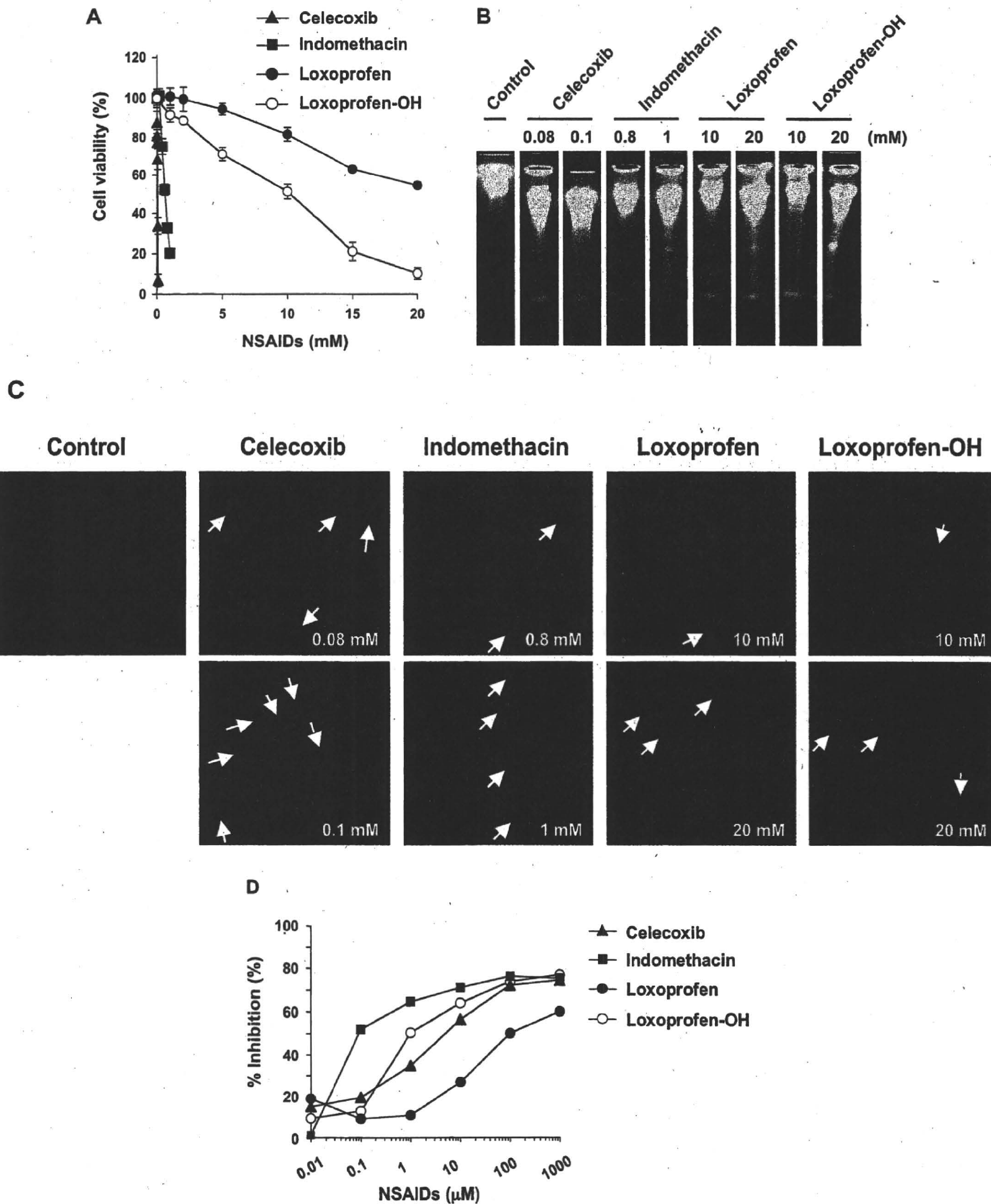

Fig. 4. Apoptosis Induced by NSAIDs in AGS Cells

AGS cells were incubated with indicated concentrations of NSAIDs for 24 h (A—C) or 4 h (D). Cell viability was determined by the MTT method. Values are mean±S.E.M. (n=3) (A). Apoptotic DNA fragmentation (B) and chromatin condensation (C) were monitored as described in Materials and Methods. The arachidonic acid (50 μM at final) was added and cells were incubated for 15 min. The amount of PGE_2 in culture medium was determined by EIA and results were shown as inhibition of COX synthesis (D).

both loxoprofen and loxoprofen-OH showed weak activity for hemolysis (Fig. 3B). Loxoprofen showed lower permeabilization activity than loxoprofen-OH on the hemolysis assay (Fig. 3B).

The results shown in Fig. 3 suggest that the low direct cytotoxicity of loxoprofen and loxoprofen-OH on gastric mucosal cells is due to their low membrane permeabilizing effects.

Cytotoxic Effects of Loxoprofen and Loxoprofen-OH on Gastric Cancer Cells In addition to their anti-inflammatory effects, recent epidemiological studies have revealed that prolonged NSAID use reduces the risk of cancer, while preclinical and clinical studies have indicated that some NSAIDs are effective in the treatment and prevention of cancer.[35] The anti-tumorigenic activity of NSAIDs is believed to involve various mechanisms, including induction of apoptosis.[36,37] Thus, it is important to examine the apoptosis-inducing ability of loxoprofen in cancer cells and we here used cultured AGS cells for this purpose.

As shown in Fig. 4A, each NSAID induced apoptosis in a dose dependent manner in AGS cells and loxoprofen and loxoprofen-OH showed less activity for inducing apoptosis than indomethacin and celecoxib. We confirmed that cell death observed in Fig. 4A is mediated by apoptosis, because it was accompanied by apoptotic DNA fragmentation and apoptotic chromatin condensation (Figs. 4B, C). Comparing to data in primary culture of gastric mucosal cells (Fig. 2), loxoprofen and loxoprofen-OH induced apoptosis more potently in AGS cells. The ED_{70} values of NSAIDs for apoptosis (concentrations of NSAIDs required for 70% cell viability by apoptosis) of loxoprofen and loxoprofen-OH were lower in AGS cells than in primary culture of gastric mucosal cells (Table 1). On the other hand, the ED_{70} values for apoptosis of indomethacin and celecoxib were nearly indistinguishable between AGS cells and primary culture of gastric mucosal cells (Table 1). Although the underlying mechanism is unknown at present, this character of loxoprofen and loxoprofen-OH may be clinically beneficial for their application as anti-tumor drugs.

We also examined the effect of NSAIDs on the COX activity in cultured AGS cells. As shown in Fig. 4D, each of all NSAIDs tested decreased the amount of PGE_2 in the culture medium, in other words, inhibited COX activity in a dose dependent manner.

In summary, we show here that loxoprofen and loxoprofen-OH have a very low level of direct cytotoxicity on gastric mucosal cells *in vitro*. As described above, it is well known that loxoprofen is clinically safe on gastric mucosa compared to other NSAIDs such as indomethacin.[25,26] We propose here that the low direct cytotoxicity of loxoprofen make it less harmful on the gastric mucosa for clinical use.

As described above, we have suggested that both COX inhibition (decrease in gastric level of PGE_2) and gastric mucosal cell death are required for the formation of NSAID-induced gastric lesions *in vivo* (Fig. 5).[20,24] Based on this idea, either NSAIDs without decreasing gastric level of PGE_2 or NSAIDs with lower cytotoxic effect should be safe NSAIDs on gastric mucosa. In other words, NSAIDs that have high cytotoxic effect on gastric mucosa and high ability to inhibit COX-1 expressed in gastric mucosa should have high risk for formation of gastric lesions. Indomethacin belongs to this type of NSAIDs and has relatively high risk for formation of gastric lesions clinically. Selective COX-2 inhibitors are relatively safe for gastric mucosa, because they have lower ability to inhibit COX-1 expressed in gastric mucosa, resulting in maintenance of gastric level of PGE_2. However, potential risk for cardiovascular thrombotic events is concern. Thus, we propose that NSAIDs with lower cytotoxic effect likely to be therapeutically beneficial NSAIDs in terms of gastrointestinal and cardiovascular safety. We are now synthesizing derivatives of loxoprofen to obtain more safe NSAIDs on gastric mucosa.

Table 1. NSAID Concentrations Required for Apoptosis

NSAIDs	Primary cell (mM)	AGS cell (mM)
Celecoxib	0.06	0.05
Indomethacin	0.42	0.40
Loxoprofen	<20	11.8
Loxoprofen-OH	19.1	4.3

ED_{70} values of NSAIDs for apoptosis (concentrations of NSAIDs required for 70% cell viability by apoptosis) in primary culture of gastric mucosal cells and in AGS cells were calculated based on results provided in Fig. 1 and Fig. 4, respectively.

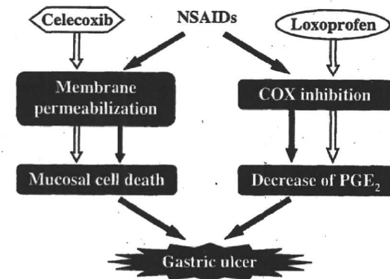

Fig. 5. A Model for Production of Gastric Lesions by NSAIDs

We have proposed that both COX inhibition (decrease in gastric level of PGE_2) and gastric mucosal cell death are required for the formation of NSAID-induced gastric lesions *in vivo*.[20,24] This idea can explain the safety of selective COX-2 inhibitors, such as celecoxib, and loxoprofen on gastric mucosa, because they have lower ability to decrease gastric level of PGE_2 and to induce gastric mucosal cell death, respectively.

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14

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Prevention of UVB Radiation-induced Epidermal Damage by Expression of Heat Shock Protein 70*[S]

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Minoru Matsuda[‡], Tatsuya Hoshino[‡], Yasuhiro Yamashita[‡], Ken-ichiro Tanaka[‡], Daisuke Maji[§], Keizo Sato[‡], Hiroaki Adachi[¶], Gen Sobue[¶], Hironobu Ihn[‡], Yoko Funasaka[‖], and Tohru Mizushima[‡1]

From the [‡]Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, [§]Saishunkan Pharmaceutical Co., Ltd., Kumamoto 861-2201, the [¶]Nagoya University Graduate School of Medicine, Nagoya 466-8550, and the [‖]Kobe University Graduate School of Medicine, Kobe 650-0017, Japan

Irradiation with UV light, especially UVB, causes epidermal damage via the induction of apoptosis, inflammatory responses, and DNA damage. Various stressors, including UV light, induce heat shock proteins (HSPs) and the induction, particularly that of HSP70, provides cellular resistance to such stressors. The anti-inflammatory activity of HSP70, such as its inhibition of nuclear factor kappa B (NF-κB), was recently revealed. These *in vitro* results suggest that HSP70 protects against UVB-induced epidermal damage. Here we tested this idea by using transgenic mice expressing HSP70 and cultured keratinocytes. Irradiation of wild-type mice with UVB caused epidermal damage such as induction of apoptosis, which was suppressed in transgenic mice expressing HSP70. UVB-induced apoptosis in cultured keratinocytes was suppressed by overexpression of HSP70. Irradiation of wild-type mice with UVB decreased the cutaneous level of IκB-α (an inhibitor of NF-κB) and increased the infiltration of leukocytes and levels of pro-inflammatory cytokines and chemokines in the epidermis. These inflammatory responses were suppressed in transgenic mice expressing HSP70. *In vitro*, the overexpression of HSP70 suppressed the expression of pro-inflammatory cytokines and chemokines and increased the level of IκB-α in keratinocytes irradiated with UVB. UVB induced an increase in cutaneous levels of cyclobutane pyrimidine dimers and 8-hydroxy-2′-deoxyguanosine, both of which were suppressed in transgenic mice expressing HSP70. This study provides genetic evidence that HSP70 protects the epidermis from UVB-induced radiation damage. The findings here also suggest that the protective action of HSP70 is mediated by anti-apoptotic, anti-inflammatory, and anti-DNA damage effects.

The skin can be structurally classified into several layers, including the most apical layer, the epidermis, containing large numbers of keratinocytes, and a second layer, immediately under this, the dermis, which has a high fibroblast content (1). Skin provides a major interface between the environment and the body and is constantly exposed to an array of physical and chemical stressors. Therefore, in addition to intrinsic causes, harmful exogenous causes are involved in the process of skin damage. Among exogenous harmful agents, UV irradiation is the most relevant to skin damage (photo-damage). UV light can be separated, based on wavelength, into three categories: UVA (320–400 nm), UVB (290–320 nm), and UVC (100–290 nm). Of these, the cell-damaging effect of UVA is relatively weak, whereas most UVC is absorbed by the ozone layer (2). Thus, UVB seems to play the central role in photo-damage, such as clinical sunburn, hyperpigmentation, erythema, plaque-like thickening, loss of skin tone, deep furrowing, and fine wrinkle formation, all of which constitute both clinical and cosmetic problems. Furthermore, UVB irradiation induces the development of skin cancer (photo-carcinogenesis) (3). UVB-induced photo-damage and photo-carcinogenesis both involve epidermal damage (such as induction of apoptosis), immunosuppression, inflammation (activation of pro-inflammatory cytokines and chemokines), and DNA damage (4). Because most UVB radiation is absorbed at the epidermis, keratinocytes become a major target of its deleterious effects. For example, the UVB-induced disruption of collagen and elastin (deep furrowing and fine wrinkle formation in the skin) involves inhibition of their synthesis in fibroblasts and stimulation of their degradation by matrix metalloproteinases and other proteases, both of which are triggered by pro-inflammatory cytokines and chemokines released from UVB-irradiated keratinocytes (4, 5). Therefore, suppression of UVB-induced damage (apoptosis) of keratinocytes is beneficial for the prevention of photo-damage. However, because such protection may actually aid in the survival of DNA-damaged cells, resulting in promotion of photo-carcinogenesis, a mechanism that not only suppresses UVB-induced apoptosis but also UVB-induced DNA damage is important to establish protocols to prevent photo-damage without promoting photo-carcinogenesis.

UVB irradiation damages the epidermis both directly and indirectly. For example, in addition to UVB-induced direct damage of nucleic acids, proteins, and lipids, UVB irradiation stimulates the production of reactive oxygen species

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[1] To whom correspondence should be addressed: Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan. Tel./Fax: 81-96-371-4323; E-mail: mizu@gpo.kumamoto-u.ac.jp.

15

(ROS),[2] which also damages these molecules by oxidization. In this way, direct absorption of UVB by DNA causes DNA damage through the formation of covalent linkages, resulting in products such as cyclobutane pyrimidine dimers (CPDs). On the other hand, UVB-produced ROS also damage DNA by producing damaged nucleotides such as 8-hydroxy-2'-deoxyguanosine (8-OHdG) (6). Supporting this notion, it was reported that anti-oxidant molecules prevent UVB-induced epidermal DNA damage (7). Thus, mechanisms that protect the epidermis from both UVB and ROS are important to establish ways in which to suppress photodamage efficiently.

When cells are exposed to stressors, a number of so-called stress proteins are induced to confer protection against such stressors. Heat shock proteins (HSPs) are representative of these stress proteins, and their cellular up-regulation of expression, especially that of HSP70, provides resistance given that HSPs re-fold or degrade denatured proteins produced by stressors such as ROS (8, 9). Because stressor-induced tissue damage is involved in various diseases, HSPs and HSP inducers have received much attention for their therapeutic potential. It is known that various HSPs are constitutively expressed in keratinocytes and their expression, especially that of HSP70, is up-regulated by different stressors (10–13). UVB irradiation of keratinocytes induces the expression of HSP70 not only *in vitro* but also *in vivo* (11, 13–17). Furthermore, artificial expression of HSP70 in keratinocytes confers protection against UVB and ROS *in vitro* (8, 16, 18, 19). The protective role of HSP70 against UVB-induced epidermal damage was also suggested by *in vivo* studies: the whole body hyperthermia of mice prevented UVB-induced sunburn cell formation, and HSP70-null mice showed a sensitive phenotype to UVB-induced epidermal damage (20–22). Protection of the skin against UVB by expression of HSP70 has been suggested to occur in human skin (21). These previous results suggest that HSP70 expression suppresses UVB-induced epidermal damage, although no genetic evidence has been reported showing that overproduction of HSP70 prevents UVB-induced epidermal damage.

The potential benefit of HSP70 inducers as medicines for UVB-related skin diseases and cosmetics was also supported by a number of previously reported observations. For example, HSP70 has an anti-inflammatory activity by means of its inhibition of nuclear factor kappa B (NF-κB) and a resulting suppression of pro-inflammatory cytokine and chemokine expression (23–26). HSP70 has been reported to stimulate base excision repair, possibly by activation of human AP endonuclease and DNA polymerase β (27–29). We also recently found that artificial overexpression of HSP70 in mouse melanoma cells suppresses melanin production.[3] Although we showed in that study that the UVB-induced production of melanin in the skin is suppressed in transgenic mice expressing HSP70, the anti-inflammatory and protective effects against DNA damage of HSP70 in UVB-irradiated skin have not been proved genetically. In this study, we examined the protective role of HSP70 against photo-damage by using transgenic mice expressing HSP70. The results obtained here suggest that expression of HSP70 protects the epidermis against UVB-induced damage via anti-inflammatory and anti-apoptotic effects and suppression of DNA damage. Based on these findings, we propose that non-toxic HSP70 inducers could be beneficial for use in cosmetics and medicines for the treatment of UVB-related skin diseases.

EXPERIMENTAL PROCEDURES

Materials and Animals—Paraformaldehyde, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), peroxidase standard and fetal bovine serum were obtained from Sigma-Aldrich. Enzyme-linked immunosorbent assay kits for interleukin (IL)-1β and IL-6 were from Pierce. Mayer's hematoxylin, 1% eosin alcohol solution, and malinol were from Muto Pure Chemicals (Tokyo, Japan). Terminal nucleotidyltransferase was obtained from Toyobo (Osaka, Japan). The Envision kit was from Dako (Carpinteria, CA). Biotin-14-ATP and Alexa Fluor 488-conjugated streptavidin were purchased from Invitrogen (Carlsbad, CA). VECTASHIELD was from Vector Laboratories. 4',6-Diamidino-2-phenylindole (DAPI) was from Dojindo Laboratories (Kumamoto, Japan). The RNeasy Fibrous Tissue Mini kit was obtained from Qiagen Inc. (Valencia, CA). The first-strand cDNA synthesis kit was from Takara Bio (Ohtsu, Japan), and IQ SYBR Green Supermix was from Bio-Rad (Hercules, CA). Lipofectamine (TM2000) and pcDNA3.1 plasmid were obtained from Invitrogen. Antibodies against IκB-α and actin were from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody against HSP70 was from Stressgen (Ann Arbor, MI). Antibody against CPDs was from Kamiya Biomedical Co. (Seattle, WA), whereas another against 8-OHdG was from Nikken SEIL (Shizuoka, Japan). α-(4-Pyridyl-1-oxide)-*N-tert*-butylnitrone (POBN) was from Alexis (San Diego, CA). Transgenic mice expressing HSP70 and their wild-type counterparts (6–8 weeks old, male) were gifts from Drs. C. E. Angelidis and G. N. Pagoulatos (University of Ioannina, Ioannina, Greece) and were prepared as described previously (30). Homozygotic transgenic mice expressing HSP70 were used in these experiments. The experiments and procedures described here were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health and were approved by the Animal Care Committee of Kumamoto University.

UV Irradiation—Animals and cultured cells were exposed to UVB irradiation with a double bank of UVB lamps (peak emission at 312 nm, VL-215LM lamp, Vilber Lourmat). The UV energy was monitored by a radiometer sensor (UVX-31, UV

[2] The abbreviations used are: ROS, reactive oxygen species; CPD, cyclobutane pyrimidine dimer; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; GGA, geranylgeranylacetone; HSP, heat shock protein; IL, interleukin; IκB-α, an inhibitor of NF-κB; MCP-1, monocyte chemoattractant protein-1; MIP-2, macrophage inflammatory protein-2; MPO, myeloperoxidase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor kappa B; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; POBN, α-(4-pyridyl-1-oxide)-*N-tert*-butylnitrone; RT, reverse transcription; TUNEL, terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling.

[3] T. Hoshino, M. Matsuda, Y. Yamashita, M. Takehara, M. Fukuya, K. Mineda, D. Maji, H. Ihn, Y. Funasaka, and T. Mizushima, unpublished data.

Products). Animals were placed under deep anesthesia with chloral hydrate (250 mg/kg), and fur was removed with electric clippers prior to the irradiation.

MPO Activity—Myeloperoxidase (MPO) activity in the skin was measured as described previously (30). Animals were placed under deep ether anesthesia and killed. The skin was dissected, rinsed with cold saline, and cut into small pieces. Samples were homogenized in 50 mM phosphate buffer, freeze-thawed, and centrifuged. The protein concentrations of the supernatants were determined using the Bradford method (31). MPO activity was determined in 10 mM phosphate buffer with 0.5 mM *o*-dianisidine, 0.00005% (w/v) hydrogen peroxide, and 20 μg of protein. MPO activity was obtained from the slope of the reaction curve, and its specific activity was expressed as the number of hydrogen peroxide molecules converted per minute/mg of protein.

Immunoblotting Analysis—Whole cell extracts were prepared as described previously (32). The protein concentration of each sample was determined by the Bradford method (31). Samples were applied to 9% (HSP70 and actin) or 12% (IκB-α) polyacrylamide SDS gels and subjected to electrophoresis, after which the proteins were immunoblotted with appropriate antibodies.

Real-time Reverse Transcription-PCR Analysis—Total RNA was extracted from skin tissues using the RNeasy Fibrous Tissue Mini kit according to the manufacturer's protocol. Samples (2.5 μg of RNA) were reverse-transcribed using the first-strand cDNA synthesis kit according to the manufacturer's instructions. Synthesized cDNA was used in real-time reverse transcription-PCR (Chromo 4 system, Bio-Rad) experiments using iQ SYBR Green Supermix and analyzed with Opticon Monitor software according to the manufacturer's instructions. Specificity was confirmed by electrophoretic analysis of the reaction products and by inclusion of template- or reverse transcriptase-free controls. To normalize the amount of total RNA present in each reaction, glyceraldehyde-3-phosphate dehydrogenase cDNA was used as an internal standard. The primers used were, *hsp70*, 5'-tggtgctgacgaagatgaag-3' (forward) and 5'-aggtcgaagatgagcacgtt-3' (reverse); *il-1β*, 5'-gatcccaagcaatacccaaa-3' (forward) and 5'-ggggaactctgcagactcaa-3' (reverse); *il-6*, 5'-ctggagtcacagaaggagtgg-3' (forward) and 5'-ggtttgccgagtagatctcaa-3' (reverse); monocyte chemoattractant protein-1 (*mcp-1*), 5'-ctcacctgctgctactcattc-3' (forward) and 5'-gcttgaggtggttgtggaaaa-3' (reverse); macrophage inflammatory protein-2 (*mip-2*), 5'-accctgccaagggttgacttc-3' (forward) and 5'-ggcacatcaggtacgatccag-3' (reverse); and *gapdh*, 5'-aactttggcattgtggaagg-3' (forward) and 5'-acacattgggggtaggaaca-3' (reverse).

Histological and Immunohistochemical Analyses and TUNEL Assay—Skin samples were fixed in 4% buffered paraformaldehyde and embedded in paraffin before being cut into 4-μm-thick sections, which were then deparaffinized and washed in phosphate-buffered saline.

For histological examination (hematoxylin and eosin staining), sections were stained first with Mayer's hematoxylin and then with 1% eosin alcohol solution. Samples were mounted with malinol and inspected using a BX51 microscope (Olympus).

For immunohistochemical analyses, sections were incubated with 0.1% (for 8-OHdG) or 0.3% (for CPDs and HSP70) hydrogen peroxide in methanol for removal of endogenous peroxidase. Sections were incubated with 0.125% trypsin in phosphate-buffered saline for 10 min and then with 1 N HCl for 30 min for DNA denaturation. Sections were blocked with 2.5% goat serum for 10 min, incubated for 12 h with antibody against HSP70 (1:200 dilution), 8-OHdG (1:100 dilution), or CPDs (1:500 dilution) in the presence of 2.5% bovine serum albumin, and then incubated for 1 h with peroxidase-labeled polymer conjugated to goat anti-mouse immunoglobulins. 3,3'-Diaminobenzidine was applied to the sections, which were then incubated with Mayer's hematoxylin (hematoxylin staining was omitted for 8-OHdG). Samples were mounted with malinol and inspected using a BX51 microscope (Olympus). The intensity of 8-OHdG staining in the epidermis was measured by LuminaVision (Mitani).

For TUNEL assay, sections were incubated first with proteinase K (20 μg/ml) for 15 min at 37 °C, then with terminal nucleotidyltransferase and biotin-14-ATP for 1 h at 37 °C, and finally with Alexa Fluor 488-conjugated streptavidin and DAPI (5 μg/ml) for 2 h. Samples were mounted with VECTASHIELD and inspected using a BX51 fluorescence microscope (Olympus).

Cell Culture and Apoptosis Analysis—PAM212 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in a humidified atmosphere of 95% air with 5% CO_2 at 37 °C. Transfection of PAM212 cells with pcDNA3.1 containing the *hsp70* gene (33) was carried out using Lipofectamine (TM2000) according to the manufacturer's protocol. The stable transfectants expressing HSP70 were selected by immunoblotting and real-time reverse transcription-PCR analyses. Positive clones were maintained in the presence of 200 μg/ml G418. Cell viability was determined by the MTT method as previously described (34), and the measurements of caspase-3-like activity and fluorescence-activated cell sorting analysis (for measurement of apoptotic cells in sub-G_1) were performed as described previously (34).

Immunostaining of 8-OHdG and CPDs in Cultured Cells—Cells were cultured on 8-well Lab-Tek II Chamber slides (Nunc). They were then fixed in methanol for 20 min after UVB irradiation. Cells were permeabilized with 0.5% Triton X-100 for 5 min, treated in a microwave oven with 0.01 M citric acid buffer for antigen activation, and then treated with 1 N HCl for 20 min for DNA denaturation. Cells were blocked with 5% goat serum for 10 min, incubated for 2 h with antibody against 8-OHdG (1:10 dilution) or CPDs (1:2000 dilution) in the presence of 2.5% bovine serum albumin, and finally incubated with Alexa Fluor 488 goat anti-mouse immunoglobulin G. Cells were simultaneously stained with DAPI (5 μg/ml) for 2 h. Samples were mounted with VECTASHIELD and inspected with the aid of a BX51 fluorescence microscope (Olympus). The fluorescence intensity of 8-OHdG or CPD staining was measured by using LuminaVision.

Determination of ROS Production in Vivo by ESR Analysis—*In vivo* ESR analysis was performed as described (35) with some modifications. Immediately after UVB exposure, animals were placed under deep anesthesia with chloral hydrate (250 mg/kg) and injected with POBN (a spin trap reagent) (36, 37) intraperitoneally (4 mmol/kg). After 1 h, mice were sacrificed, the skins

were dissected, and the lipid phase was extracted. After evaporating the sample, ESR spectra were immediately recorded at room temperature using a quartz flat cell (160 μl) in a JES-TE200 spectrometer (JEOL). The operating conditions of the ESR apparatus were: 9.43 GHz, field 335.2 ± 5 milliteslas, 40-milliwatt microwave power, 100-kHz modulation frequency, 0.25-field modulation width, 0.3-s time count, and sweep time of 2 min.

Statistical Analysis—All values are expressed as the means ± S.E. Two-way analysis of variance followed by the Tukey test was used to evaluate differences between more than three groups. Differences were considered to be significant for values of $p < 0.05$.

RESULTS

Effect of Expression of HSP70 on UVB-induced Epidermal Apoptosis—Overexpression of HSP70 in the transgenic mice that we used in this study has been shown in various organs (9, 30, 38–40). We examined HSP70 expression in the skin of these animals as this has not been determined to date. Transgenic mice expressing HSP70 and wild-type mice were irradiated or not with 180 mJ/cm² UVB. The dorsal skin was removed 24 h after completion of the irradiation and subjected to immunoblotting analysis. As shown in Fig. 1 (*A* and *B*), the level of [illegible] than in wild type mice in both the presence and absence of UVB irradiation. However, under these conditions, UVB irradiation did not up-regulate the expression of HSP70 in either type of mice (Fig. 1, *A* and *B*), a finding that differs from previous reports (20). Although we examined the effect of UVB on expression of HSP70 under various conditions (various doses of UVB and time course of the induction periods), we could not detect the UVB-dependent up-regulation of expression of HSP70 under any conditions by immunoblotting analysis (supplemental Fig. S1). We consider that this is due to the UVB-dependent increase in total amount of proteins (we applied the same amount of proteins in each lane in immunoblotting analysis). Supporting this notion, immunohistochemical analysis with an antibody against HSP70 demonstrated that the expression of HSP70 was induced by UVB irradiation at the skin (the *top panels* in supplemental Fig. S2). Immunohistochemical analysis also demonstrated that the expression of HSP70 is higher in the epidermis than in the dermis, as described previously (11), and that expression in the epidermis is further heightened in transgenic mice (Fig. 1*C*). The results in Fig. 1 suggest that these transgenic mice could be useful for examining the protective role of HSP70 against UVB-induced epidermal damage.

Histological observations revealed extensive infiltration of leukocytes and epidermal disruption in skin sections prepared from UVB-irradiated wild-type mice, whereas the extent of cutaneous damage was not so apparent in transgenic mice expressing HSP70 (Fig. 2*A*). MPO activity, an indicator of the inflammatory infiltration of leukocytes, was increased in wild-type mice in response to the UVB irradiation. This activity was lower in UVB-irradiated transgenic mice expressing HSP70 compared with wild-type mice (Fig. 2*B*). The overexpression of HSP70 in transgenic mice did not affect the background level of MPO activity (Fig. 2*B*). These results show that UVB-induced

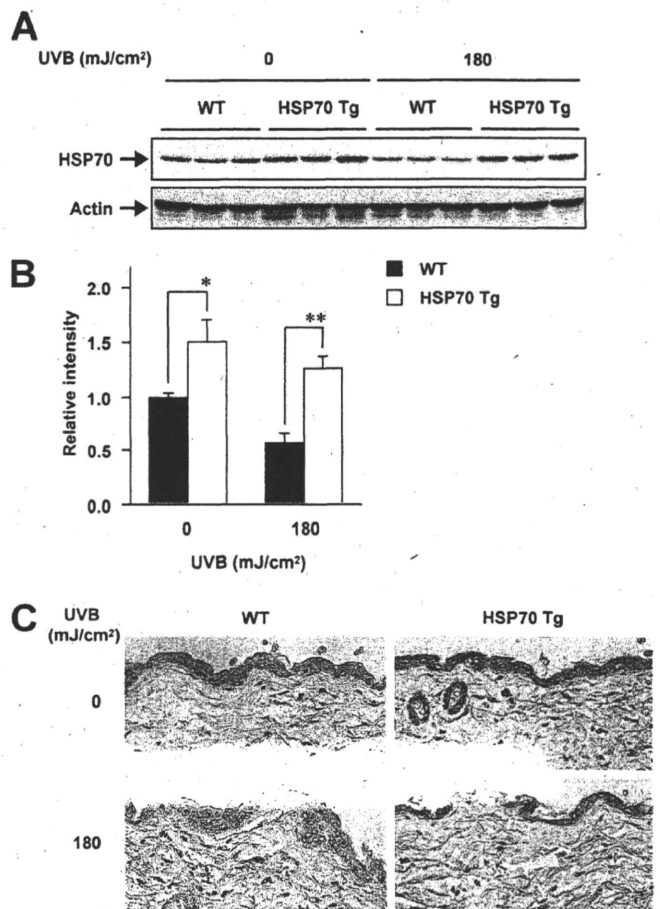

FIGURE 1. **Expression of HSP70 in the dorsal skin after UVB irradiation.** Transgenic mice expressing HSP70 (*HSP70 Tg*) and wild-type mice (*WT*) were irradiated with or without 180 mJ/cm² UVB, and the dorsal skin was removed after 24 h. *A*, whole cell extracts were analyzed by immunoblotting with an antibody against HSP70 or actin. *B*, the band intensity of HSP70 was determined and expressed relative to the control sample ($n = 6$) (one of two gels is shown in *panel A*). Values are mean ± S.E. **, $p < 0.01$; *, $p < 0.05$. *C*, sections of dorsal skin were prepared and subjected to immunohistochemical analysis with an antibody against HSP70. *Brown* staining indicates HSP70 expression. *Scale bar*, 50 μm.

epidermal damage and the resulting infiltration of leukocytes are suppressed in transgenic mice expressing HSP70.

The extent of epidermal cell apoptosis was determined by TUNEL assay. An increase of TUNEL-positive (apoptotic) cells in the epidermis of wild-type mice was observed after the UVB irradiation, and this increase was clearly suppressed in transgenic mice expressing HSP70 (Fig. 2, *C* and *D*). The overexpression of HSP70 in transgenic mice did not affect the background level of epidermal apoptosis (Fig. 2, *C* and *D*). These results suggest that the expression of HSP70 protects epidermal cells (keratinocytes) from UVB-induced apoptosis.

To identify cells expressing HSP70 in transgenic mice and wild-type mice irradiated with UVB, we performed co-immunostaining assay. As shown in supplemental Fig. S2, strong co-staining of HSP70 with CD11b (a marker of macrophage) and pan cytokeratin (a marker of keratinocyte) was observed at the skin of transgenic mice expressing HSP70 or wild-type mice irradiated with UVB. A relatively weak co-staining of HSP70 with MPO (a maker of neutrophil) and vimentin (a maker of fibroblast) was also observed (supplemental Fig. S2). These

18