Inhibitory Effect of β-Thujaplicin on Ultraviolet B-Induced Apoptosis in Mouse Keratinocytes

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Sunburn cells are thought to represent ultraviolet B-induced apoptotic keratinocytes. It has been demonstrated that enzymatic and nonenzymatic antioxidants effectively suppress sunburn cell formation, indicating that reactive oxygen species may play a role in the progression of ultraviolet B-induced apoptosis. Metallothionein, a cytosol protein, has antioxidant activity, and overexpression of metallothionein has been reported to reduce the number of sunburn cells in mouse skin. We have also demonstrated that overexpression of metallothionein inhibits ultraviolet B-induced DNA ladder formation in mouse keratinocytes. These findings support the hypothesis that cellular metallothionein may play an important role in the inhibition of ultraviolet B-induced apoptosis in keratinocytes through its antioxidant activity. In the present study, we investigated the effects of β-thujaplicin, an extract from the woods of *Thuja plicata D. Don*. and *Chamaecyparis obtuse*, *Sieb. et Zucc*., on ultraviolet B-induced apoptosis in keratinocytes and on metallothionein induction. Topical application of β-thujaplicin decreased the number of ultraviolet B-mediated sunburn cells and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling-positive cells in mouse ear skin. Incubation with β-thujaplicin suppressed ultraviolet B-induced DNA ladder formation in cultured mouse keratinocytes. Histochemical analysis showed that topical application of β-thujaplicin induced metallothionein protein in mouse skin. Northern analysis and western blotting revealed significant induction of metallothionein mRNA and metallothionein protein, respectively, in β-thujaplicin-treated cultured mouse keratinocytes. These findings indicate that β-thujaplicin inhibits ultraviolet B-induced apoptosis in keratinocytes and strongly suggest that the inhibitory mechanism is due to the antioxidant activity of metallothionein induced by the agent. ***Key words: metallothionein/reactive oxygen species. J Invest Dermatol 110:24–28, 1998***

Sunburn cells (SBC) that appear in the epidermis *in vivo* after ultraviolet B (UVB) irradiation (Woodcock and Magnus, 1976; Danno and Horio, 1987) are thought to be UVB-induced apoptotic cells (Kerr and Wyllie, 1972; Kane and Maytin, 1995; Baba *et al*, 1996). Treatment with enzymatic (Miyachi *et al*, 1983; Danno *et al*, 1984) and nonenzymatic (Mathews-Roth, 1986; Hanada *et al*, 1997) antioxidants inhibits SBC formation in mouse skin. These findings suggest that reactive oxygen species (ROS) may play a role in the progression of UVB-induced apoptosis.

Metallothionein (MT) is a cytosol protein with a low molecular weight (6.0–7.0 kDa) and a high cysteine content, which has the capacity to selectively bind heavy metals and scavenge ROS within cells. MT has antioxidant activity against hydroxyl and superoxide radicals (Thornalley and Vasac, 1985; Hamer, 1986; Hanada *et al*, 1992/1993). One of the present authors has reported that the overexpression of MT induced by cadmium chloride (Cd^{2+}), which is a potent inducer of MT, reduces the number of SBC in mouse skin (Hanada *et al*, 1991). In this study, we demonstrated that Cd^{2+} treatment inhibited the UVB-induced apoptotic process in mouse keratinocytes by observation of DNA ladder formation. These findings support the hypothesis that cellular MT may play an important role in inhibiting UVB-induced apoptosis in keratinocytes through its antioxidant activity.

β-Thujaplicin (Hinokitiol) is a tropolone-related compound purified from the wood of *Thuja plicata D. Don*. and *Chamaecyparis obtuse*, *Sieb. et Zucc*. (Nozoe, 1936; Erdtman and Gripenberg, 1936). β-Thujaplicin is known to have biologic activities, including anti-bacterial, anti-fungal (Anderson and Gripenberg, 1948), anti-tumor (Yamato *et al*, 1984), and antioxidant actions (Crawford and Schneider, 1982; Arima *et al*, 1997). Our interest was focused on the fact that β-thujaplicin is a scavenger for hydroxyl radicals and hydrogen peroxide. Recently, we found inducible activity of β-thujaplicin for MT. This led us to hypothesize that in addition to Cd^{2+}, β-thujaplicin may suppress UVB-induced apoptosis in keratinocytes. Here we report our studies on the induction of MT by β-thujaplicin and the possible inhibitory effect of the agent on UVB-induced apoptosis in mouse keratinocytes.

MATERIALS AND METHODS

Animals Eight female BALB/C mice, aged 6–8 wk, were obtained from Japan Clea Laboratories (Tokyo, Japan). The animals were kept under standard conditions with free access to food and water.

Cell culture The cultured mouse keratinocyte line, PAM212 (kindly provided by Dr. K. Adachi, Shiseido Research Center, Yokohama, Japan), which had undergone spontaneous malignant transformation in culture (Yuspa *et al*, 1980), was used. The cells were seeded in dishes and cultured at 37°C under 5% CO_2 in Eagle's minimal essential medium (Gibco, Grand Island, NY) containing

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Abbreviations: MT, metallothionein; SBC, sunburn cell; SOD, superoxide dismutase; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling.

10% fetal calf serum (Gibco), 50 U penicillin per ml, 50 μg streptomycin per ml, 2.5 μg amphotericin B per ml, and 2 mg $NaHCO_3$ per ml. Cells were used for experiments under subconfluent conditions.

Reagents β-Thujaplicin (Takasago, Tokyo, Japan) was dissolved in 5 mM L-arginine solution (Sigma, St. Louis, MO) at concentrations of 50, 100, 250, and 500 μM. The major peak of absorption on the spectrum of β-thujaplicin/L-arginine solution was observed at a wavelength of 242 nm, with minor absorption peaks at 332.2 nm and 387 nm. β-Thujaplicin in purified petrolatum revealed maximum absorption at 216 nm. No absorption was observed within the UVB range.

UVB irradiation source A bank of seven closely set FL20S E-30 fluorescent sunlamps (Toshiba Medical Supply, Tokyo, Japan), emitting rays between 275 and 375 nm with a peak at 305 nm, and mounted in a reflective housing (DERMARAY®, Clinical Supply, Tokyo, Japan), was used for irradiation. The radiation dose was measured by a 305/365.D (II) radiometer® (Topcon, Tokyo, Japan). A long-wave length filter (Kodacel®, Kodak, Tokyo, Japan) was used for screening out contaminating ultraviolet C radiation.

Analysis of cell viability PAM212 cells were incubated with Eagle's minimal essential medium containing each concentration of β-thujaplicin (5, 10, 25, and 50 μM). Twenty-four hours after incubation, these cells were harvested and then resuspended in propidium iodide solution (Sigma) (propidium iodide 20 μg per ml in phosphate-buffered saline). Nonviable cells were determined by flow-cytometric analysis using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). The mean viability of cells in triple measurements was taken for each concentration of β-thujaplicin.

UVB irradiation of cultured cells after β-thujaplicin or Cd^{2+} treatment PAM212 cells were incubated with Eagle's minimal essential medium containing β-thujaplicin (5, 10, 25, 50 μM) and 10 μM Cd^{2+} (Wako Pure Chemicals, Osaka, Japan). Twenty-four hours after incubation, the cells were washed twice with phosphate-buffered saline (pH 7.2) to remove the agents, and irradiated with UVB at 50 mJ per cm^2 in the presence of phosphate-buffered saline. The distance from the light source was 20 cm and the exposure time was 3.5 min. After irradiation, the cells were cultured again in Eagle's minimal essential medium for 24 h, and then harvested for analysis of DNA fragmentation *in vitro*.

UVB irradiation of mouse skin after application of β-thujaplicin Before each procedure, the mice were anesthetized by intraperitoneal injection of chloral hydrate (2.9 g per kg body weight). β-Thujaplicin in purified petrolatum at a concentration of 2 mM was applied to the upper surface of the ear and occluded with pliable plastic film. As a control, purified petrolatum was applied to the other ear. After 24 h, the ointment was removed and the mouse ears were irradiated with UVB (150 mJ per cm^2). The distance from the light source to the ear was 20 cm and the exposure time was 10.5 min. Twenty-four hours after irradiation, a 5 mm punch biopsy was taken from each ear. The skin samples were fixed with 10% buffered formalin and embedded in paraffin. Paraffin sections (6 μm thick) were prepared for SBC counting and for analysis of DNA fragmentation *in vivo*.

Analysis of DNA fragmentation The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) technique (Yael *et al*, 1992) was performed for analysis of DNA fragmentation in mouse skin. In this experimental system, we used an apoptosis detection kit (ApopTag®, Oncor, Gaitheerburg, MD) and TdT enzyme solution was used at 100 fold dilution. For analysis of DNA fragmentation in cultured keratinocytes, agarose gel electrophoresis was performed. DNA was isolated from the cells using a DNA isolation kit (Sepa Gene®, Sankojunyaku, Tokyo, Japan). Twenty μg of isolated DNA treated with RNase A (10 μg per ml) (Funakoshi, Tokyo, Japan) was subjected to 2% agarose gel electrophoresis. Details of the conditions used for the TUNEL technique and agarose gel electrophoresis have been described previously (Baba *et al*, 1996).

SBC and TUNEL-positive cell counting For SBC counting, ear skin samples were stained routinely with hematoxylin and eosin for histologic examination. The histologic criteria for identification of SBC were those described by Woodcock and Magnus (1976). The numbers of SBC and TUNEL-positive cells were counted using a light microscope. The mean counts of these cells in five sections from each specimen were expressed as cells per linear centimeter of epidermis. Eight separate experiments were performed. Student's t test was used to determine the significance of differences at a p value of <0.05.

Analysis of MT expression For the *in vitro* experiment, PAM212 cells were incubated with Eagle's minimal essential medium containing β-thujaplicin at concentrations of 10 and 50 μM, or 10 μM Cd^{2+}. L-arginine was added to control medium (500 μM). Twenty-four hours after incubation, these cells were used for northern analysis and immunoblot analysis for observation of MT induction. For the *in vivo* experiment, using β-thujaplicin-treated mouse ear skin, histochemical staining was carried out with a 1:50 dilution of monoclonal mouse anti-MT antibody (Dako, Carpinteria, CA). The conditions for histochemical staining have been described previously (Hanada *et al*, 1992/1993).

RNA isolation and northern analysis RNA was isolated from the cells by a single-step extraction procedure (Chomzynski and Sacchi, 1987). Thirty μg of total RNA per lane was fractionated on 1.5% agarose gel and transferred to nitrocellulose filters, and northern hybridization was performed with a 0.5 kb human MT-II cDNA (American Type Culture Collection, Rockville, MD) radioactively labeled with [32P]CTP by nick translation (Sambrook *et al*, 1989). As a constitutively expressed control, a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (Fort *et al*, 1985) was used for hybridization. The intensity of the autoradiographic bands was quantitated by scanning densitometry of X-ray films at 633 nm, and the MT mRNA steady-state levels were corrected by the levels of GAPDH mRNA transcripts in the same RNA preparations.

Immunoblot analysis The cells were harvested and disrupted in a solution of 10 mM Tris-HCl, pH 7.8, containing 0.2 M NaCl by sonication for 60 s using a model UR-20P sonifier (Tomy Seiko, Tokyo, Japan), and centrifuged at 16,000 × *g* for 15 min. Samples of the resulting supernatants were used for immunoblot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with 15% polyacrylamide gels (Laemmli, 1970). The proteins were then transferred electrophoretically to nitrocellulose. Immunoblotting was carried out using a 1:50 dilution of monoclonal mouse anti-MT antibody (Dako) (Towbin *et al*, 1979) and visualized using 4-chlor-1-naphthol. As a positive control, MT II (Sigma) protein purified from rabbit Iiver was used.

RESULTS

Viability of PAM212 cells after treatment with β-thujaplicin To examine the cytotoxic effect of β-thujaplicin on PAM212 cells, a cell survival test was performed. The viability of the cells treated with each concentration of β-thujaplicin was over 85% (data was not shown).

Suppressive effect of β-thujaplicin on SBC formation and TUNEL-positive cell induction in mouse skin To examine the effect of β-thujaplicin on UVB-induced apoptosis *in vivo*, we observed SBC formation and TUNEL-positive cell induction. The nontreated ear skin showed a marked increase in the numbers of SBC and TUNEL-positive cells after UVB irradiation at 150 mJ per cm^2. The numbers of SBC and TUNEL-positive cells in the β-thujaplicin-treated skin were reduced by about 40% ($p < 0.01$) and 43% ($p < 0.001$), respectively, as compared with those in nontreated skin. There was no significant affect on SBC and TUNEL-positive cell formation in skin that had been left unirradiated but treated with β-thujaplicin (**Figs 1**, **2**).

Inhibitory effect of β-thujaplicin and Cd^{2+} on UVB-induced DNA ladder formation in cultured cells To examine the effect of β-thujaplicin and Cd^{2+} on UVB-induced apoptosis *in vitro*, we observed DNA fragmentation in PAM212 cells. The characteristic ladder formation was recognized in the isolated DNA obtained from the cells that were irradiated at a UVB dose of 50 mJ per cm^2 (**Fig 3**, *lane 2*). Treatment with β-thujaplicin prior to UVB irradiation suppressed DNA ladder formation in a concentration-dependent manner. Fifty μM of β-thujaplicin (**Fig 3**, *lane 5*) and 10 μM Cd^{2+} (**Fig 3**, *lane 6*) suppressed ladder formation almost completely.

Inductive effect of β-thujaplicin on MT mRNA in cultured cells To clarify the mechanism of the inhibitory effect of β-thujaplicin on UVB-induced apoptosis in keratinocytes, we examined the effect of β-thujaplicin on MT mRNA expression. Twenty-four hours of incubation with 50 μM β-thujaplicin markedly increased MT mRNA expression in PAM212 cells (**Fig 4*A***). After calibration with the GAPDH level, 50 μM β-thujaplicin had doubled the inducing effect of 10 μM Cd^{2+} on the amount of MT mRNA (**Fig 4*B***).

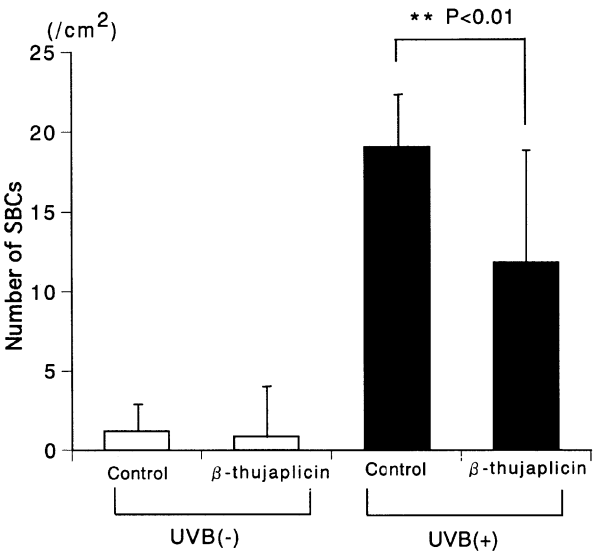

Figure 1. β-Thujaplicin suppression of SBC formation in UVB-irradiated mouse skin. Mouse ear skin was biopsied, stained with hematoxylin and eosin, and observed by light microscopy. About 40% reduction in the number of SBC was observed in the β-thujaplicin-treated mouse ear skin as compared with nontreated skin ($p < 0.01$). β-Thujaplicin treatment did not affect SBC formation in nonirradiated ear skin. Error bars represent the mean of eight separate experiments ± SD.

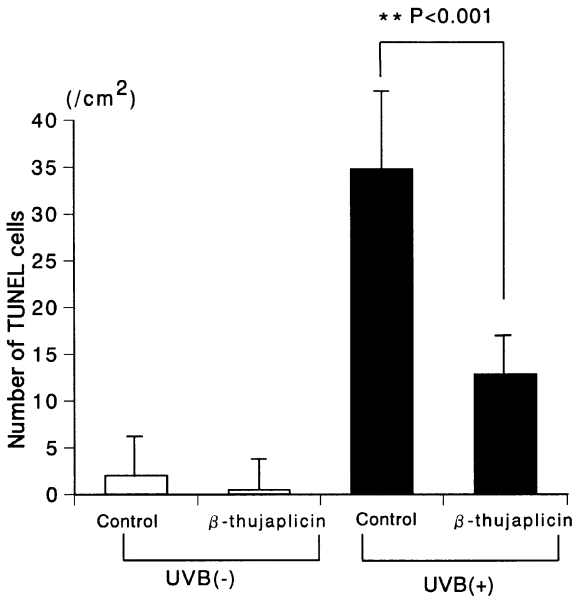

Figure 2. β-Thujaplicin suppression of TUNEL-positive cell induction in UVB-irradiated mouse skin. Mouse ear skin was biopsied, stained by the TUNEL technique, and observed by light microscopy. The number of TUNEL-positive cells was reduced by 43% ($p < 0.001$) as compared with nontreated skin. β-Thujaplicin treatment did not affect positive cell formation in nonirradiated ear skin. Error bars represent the mean of eight separate experiments ± SD.

Inductive effect of β-thujaplicin on MT protein expression We observed the effect of β-thujaplicin on the expression of MT protein. Histochemical staining showed that the expression of MT protein was upregulated in mouse skin (**Fig 5**). Immunoblot analysis revealed increased MT protein in cultured mouse keratinocytes incubated with 50 μM β-thujaplicin as well as with Cd^{2+} (**Fig 6**).

DISCUSSION

ROS have been shown to play a role in the progression of apoptosis in many different model systems. Direct exposure of HL-60 cells to hydrogen peroxide induces apoptosis in the cells (Lennon *et al*,

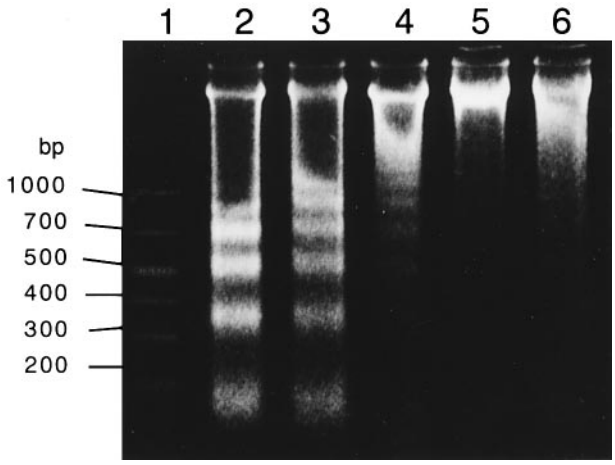

Figure 3. β-Thujaplicin and Cd^{2+} inhibition of UVB-induced DNA ladder formation in cultured mouse keratinocytes. DNA extracted from the cells was electrophoresed on 2% agarose gel. DNA ladder formation was observed 24 h after UVB irradiation at 50 mJ per cm^2 (*lane 2*). Treatment with β-thujaplicin (*lane 3*, 10 μM; *lane 4*, 25 μM; *lane 5*, 50 μM) and 10 μM Cd^{2+} (*lane 6*) prior to UVB irradiation suppressed ladder formation in a concentration-dependent manner (*lanes 3*, *4*, and *5*) (*lane 1*, size marker).

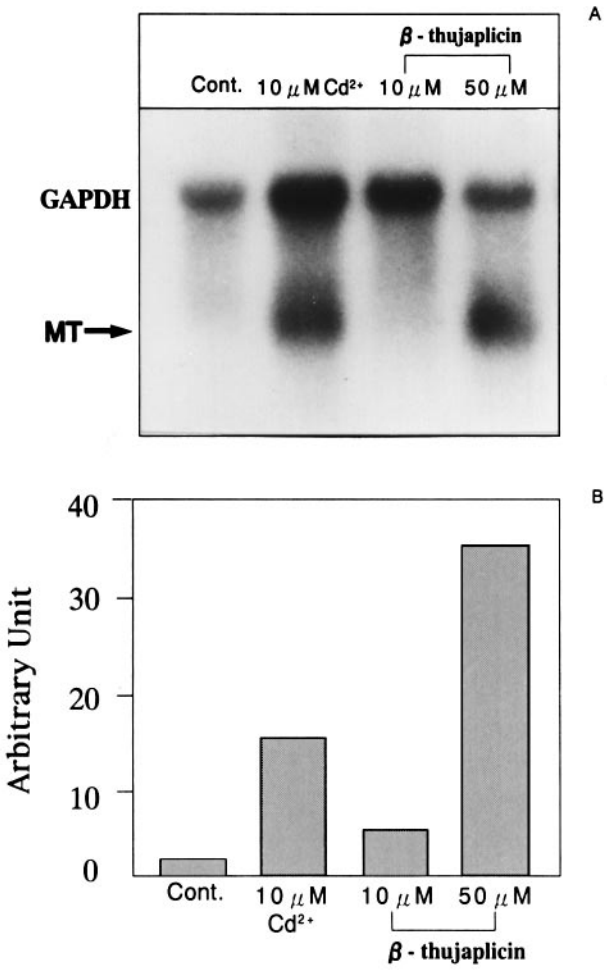

Figure 4. Increase in expression of MT mRNA induced by β-thujaplicin. Thirty micrograms of total RNA per lane was fractionated on 1.5% agarose gel, transferred to nitrocellulose filters, and northern hybridization was performed with a 0.5 kb human MT-II cDNA (*A*). The filters were hybridized to GAPDH cDNA, and the bands were scanned with a densitometer (*B*). The induction level of MT mRNA with 50 μM β-thujaplicin was double that in the presence of 10 μM Cd^{2+} (*B*).

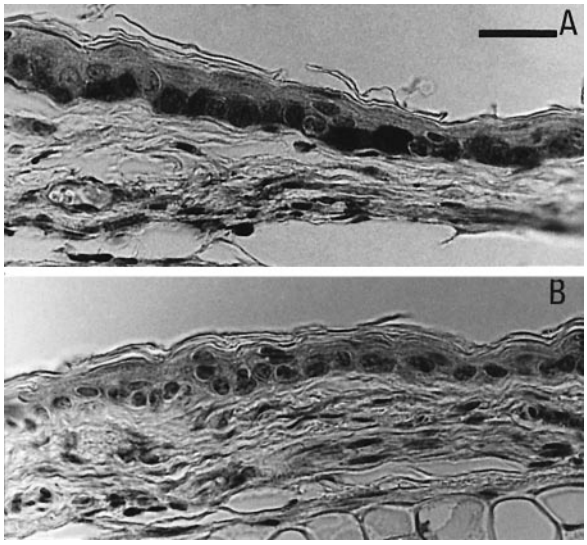

Figure 5. Increase in expression of MT protein induced by β-thujaplicin in mouse skin. Twenty-four hours after treatment with β-thujaplicin, the skin was biopsied and stained immunohistochemically using a monoclonal mouse anti-MT antibody. In the treated skin (*A*), MT expression was increased in comparison with nontreated skin (*B*). *Scale bar*, 25 μm.

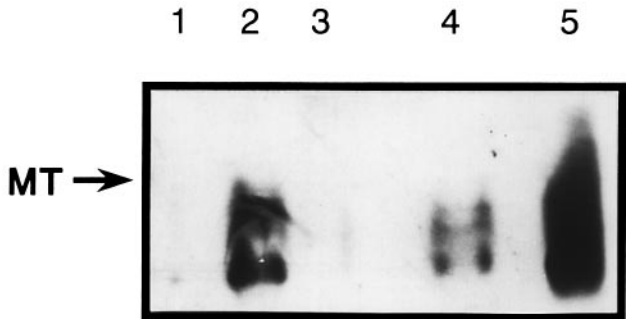

Figure 6. Increase in expression of MT protein induced by β-thujaplicin in cultured mouse keratinocytes. Epidermal proteins were separated on 15% sodium dodecyl sulfate-polyacrylamide gel, transferred electrophoretically to nitrocellulose, and reacted with a monoclonal mouse anti-MT antibody. Shown are the cells treated with Cd^{2+} (*lane 2*), 10 μM β-thujaplicin (*lane 3*), 50 μM β-thujaplicin (*lane 4*), or 500 pg MT-II protein (*lane 5*) and nontreated cells (*lane 1*, control). Immunoblot analysis demonstrated an increase of MT protein expression in the cells incubated with 10 μM and 50 μM β-thujaplicin (*lanes 3* and *4*).

1991), and other oxidants such as oxidized lipoproteins and lipid hydroperoxides induce apoptosis in an HIV-infected human T-cell line (Sandstrom *et al*, 1994). Furthermore, several antioxidants have been shown to prevent apoptosis. Apoptosis in fibroblasts induced by tumor necrosis factor-α and in thymocytes induced by dihydrolipoic acid can be prevented by various thiol-containing antioxidants such as N-acetyl cysteine (Mayer and Noble, 1994; Bustamante *et al*, 1995). Intracellular expression of superoxide dismutase (SOD) is able to inhibit apoptosis by calcium ionophore in neural cells (Rabizadeh *et al*, 1995). Downregulation of copper/zinc SOD with anti-sense oligonucleotides induces apoptosis in cultured rat pheochromocytoma cells (Troy and Shelanski, 1994). Overexpression of bcl-2, a proto-oncogene, prevents hydrogen peroxide-induced apoptosis through its antioxidant pathway in murine pro-B lymphocytes (Hockenbery *et al*, 1993). These anti-apoptotic effects of antioxidants corroborate the hypothesis that apoptotic signaling is mediated by ROS (Hockenbery *et al*, 1993; Troy and Shelanski, 1994; Sarafian and Bredesen, 1994).

In the skin, intravenous administration of SOD (Danno *et al*, 1984) and esterified GSH (Hanada *et al*, 1997) has been demonstrated to suppress UVB-induced SBC formation in mouse skin. Another study has shown that addition of SOD to culture media during and after UVB irradiation suppresses SBC formation in organ cultures of mouse skin (Miyachi *et al*, 1983). Collectively, these data indicate that ROS generated by UVB irradiation are apparently responsible for UVB-induced apoptosis in keratinocytes.

In this study we showed that β-thujaplicin inhibited UVB-induced apoptosis of keratinocytes *in vivo* and *in vitro*. In addition, we found that β-thujaplicin was a potent inducer of MT in the keratinocytes *in vivo* and *in vitro*. The inductive capacity of β-thujaplicin in expression of the MT gene and MT protein in keratinocytes has not been reported so far. Besides its binding activity with heavy metals such as cationic zinc and cadmium, MT has scavenging activity for ROS. It has been demonstrated that Cd^{2+} increases the expression of MT mRNA in cultured keratinocytes, and that systemic administration of Cd^{2+} reduces the number of SBC in UVB-irradiated mouse skin (Hanada *et al*, 1991). In this study, we showed that Cd^{2+} inhibited DNA ladder formation in UVB-irradiated keratinocytes, suggesting a possible role of cellular MT in suppression of UVB-induced apoptosis through its radical scavenging activity. Our additional experiment showed that exgenous administration of a high dose of MT failed to reduce UVB-induced DNA ladder formation (data not shown), suggesting that MT proteins function only in cytosole and that exgenously administered MT might not be transported into the cells. Altogether, these findings strongly suggest that the inhibitory mechanism of β-thujaplicin on UVB-induced apoptosis is due to the antioxidant activity of β-thujaplicin-induced MT.

β-Thujaplicin has been reported to have scavenging activity for hydroxyl radicals and hydrogen peroxide (Arima *et al*, 1997). The electron spin resonance spin-trapping technique showed that treatment with β-thujaplicin selectively suppressed the signal intensity of hydroxyl radicals generated by the Fenton reaction, and the chemiluminescence technique showed that β-thujaplicin significantly reduced the luminescence induced by hydrogen peroxide. This evidence led us to the hypothesis that the direct antioxidant activity of β-thujaplicin may play a role in this anti-apoptotic effect; however, as we removed this agent as completely as possible before UVB irradiation, we believe that the anti-apoptotic effect of β-thujaplicin observed in our experimental system is probably due to its indirect antioxidant activity through induced MT.

Other than its anti-bacterial or anti-mycotic activity, β-thujaplicin may provide a new strategy for prevention of UVB-induced apoptosis and skin damage, and therefore further study of this agent seems warranted.

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