
Protective Role of Copper, Zinc Superoxide Dismutase Against UVB-Induced Injury of the Human Keratinocyte Cell Line HaCaT

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On the basis of our recent observation that copper, zinc-superoxide dismutase and manganese-superoxide dismutase change differently following a single exposure to ultraviolet-B irradiation in the human keratinocyte cell line HaCaT, we have examined the possible role of endogenous copper,zinc-superoxide dismutase or manganese-superoxide dismutase against ultraviolet-B-induced reactive-oxygen-species-mediated keratinocyte injury *in vitro*. To evaluate the individual defensive roles of copper, zinc-superoxide dismutase and manganese-superoxide dismutase, we treated HaCaT cells with diethyldithiocarbamate, a chelating agent of ionic copper that inactivates copper,zinc-superoxide dismutase activities, tumor necrosis factor α , which enhances manganese-superoxide dismutase levels, or transforming growth factor β_1 , which inhibits manganese-superoxide dismutase levels. After the treatment with each reagent, HaCaT cells in the three different conditions were exposed to a single dose of ultraviolet-B irradiation. We assessed ultraviolet-B-induced cytotoxicity by measuring both lactate dehydrogenase leakage and cell viability using

trypan blue dye exclusion assay. The lactate dehydrogenase leakage in the supernatant from damaged HaCaT cells whose copper,zinc-superoxide dismutase levels were inactivated by diethyldithiocarbamate was significantly increased and the cell viability was significantly decreased in comparison with untreated groups at 8 and 24 h after ultraviolet-B irradiation. On the other hand, the lactate dehydrogenase release and cell viability for HaCaT cells whose manganese-superoxide dismutase levels were enhanced by tumor necrosis factor α or inhibited by transforming growth factor β_1 showed no significant difference from untreated groups. Furthermore, increased production of intracellular peroxides in HaCaT cells treated with diethyldithiocarbamate was observed by flow cytometric analysis at 8 h after ultraviolet-B irradiation. These results suggest that copper,zinc-superoxide dismutase may play a primary protective role against ultraviolet-B-induced injury of the human keratinocyte cell line HaCaT. **Key words:** diethyldithiocarbamate/lipid peroxides/reactive oxygen species/TNF- α /TGF- β_1 . *J Invest Dermatol* 114:502-507, 2000

It is generally accepted that the skin is especially vulnerable to damage by reactive oxygen species (ROS) generated in a number of physical and biological processes. Photochemical reactions with ultraviolet (UV) light as well as ionizing radiation produce ROS such as O_2^- (Carraro and Pathak, 1988). Formation of ROS in the epidermis by UVB irradiation (Darr and Fridovich, 1994; Jurkiewicz and Buettner, 1994) causes oxidative damage to cellular membrane lipids, proteins, and nucleic acids (Breimer, 1991; Tyrrell, 1995), and it is thought to be involved in various acute or chronic cutaneous changes such as erythema, sunburn cell formation (Danno *et al*, 1984; Yoshioka *et al*, 1987), photocarcinogenesis, and photoaging (Ananthaswamy and

Pierceall, 1990; Miyachi, 1995). Because keratinocytes are located in the outermost surface of the skin, they have a strong antioxidant potential and contain specific enzymes that act directly or indirectly on ROS such as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^\cdot) (Fuchs *et al*, 1989).

In particular, superoxide dismutase (SOD), which is one of the cellular antioxidant enzymes, may play a key role as a defensive mechanism against oxidative damage, because SOD catalyzes dismutation of O_2^- to O_2 and H_2O_2 (Black, 1987; Trenam *et al*, 1992), resulting in inhibition of the Haber-Weiss reaction that generates OH^\cdot mediated by O_2^- -induced metal ion reduction for the H_2O_2 -dependent Fenton's reaction (Liochev and Fridovich, 1993). OH^\cdot formation through the Haber-Weiss reaction is thought to be the most reactive and dangerous to cellular components. Two types of SOD, copper,zinc-SOD (Cu,Zn-SOD) and manganese-SOD (Mn-SOD), have been identified in mammalian cells, and keratinocytes have been reported to contain both isozymes of SOD (Kobayashi *et al*, 1991, 1993). The isozymes are encoded by two separate genes and differ in primary structure, biological features, and intracellular localization (Scherman *et al*, 1983; Ho and Crapo, 1988; Kobayashi *et al*, 1991, 1993). The two isozymes of SOD possess substantially different physiological roles

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Abbreviations: Cu,Zn-SOD, copper, zinc-superoxide dismutase; DCFH-DA, 2',7'-dichlorofluorescein diacetate; DDC, diethyldithiocarbamate; LDH, lactate dehydrogenase; Mn-SOD, manganese-superoxide dismutase; O_2^- , superoxide anion radical; OH^\cdot , hydroxyl radical; SOD, superoxide dismutase.

and are associated with different disorders in living systems. We have recently demonstrated that they change differently following UVB irradiation: Cu,Zn-SOD increases immediately after a single exposure to UVB in cultured human keratinocytes, whereas Mn-SOD decreases following UVB irradiation, thereafter gradually recovering to the control level (Sasaki *et al*, 1997). These results suggest that the two isozymes of SOD may play different defensive roles against UVB-induced oxidative stress. The aim of this study was to investigate the individual possible roles of endogenous Cu,Zn-SOD or Mn-SOD against acute phase UVB-induced injury of the human keratinocyte cell line HaCaT. We examined lactate dehydrogenase (LDH) leakage in the supernatant from damaged keratinocytes and also cell viability when Cu,Zn-SOD or Mn-SOD levels were inhibited or enhanced using diethyldithiocarbamate (DDC), tumor necrosis factor α (TNF- α), or transforming growth factor β_1 (TGF- β_1); we analyzed intracellular peroxides by flow cytometry following acute UVB irradiation, taken both as indicators of UVB-induced ROS-mediated cytotoxicity.

MATERIALS AND METHODS

Chemicals DDC, 6-hydroxypurine (hypoxanthine), ferricytochrome c (type III), and buttermilk xanthine oxidase were obtained from Sigma, (St Louis, MO). Recombinant human TNF- α and TGF- β_1 were purchased from Genzyme (Cambridge, MA). 2',7'-dichlorofluorescein diacetate (DCFH-DA) was obtained from Molecular Probes (Eugene, OR).

Cell preparations The human keratinocytes utilized in this study were HaCaT cells (Boukamp *et al*, 1988), transformed human keratinocytes provided by Professor N.E. Fusenig. HaCaT cells were cultured in Dulbecco's modified Eagle's medium (Nikken Biomedical Laboratory, Kyoto, Japan), supplemented with 5% fetal bovine serum (ICN Biomedicals, Costa Mesa, CA), a 2% mixture of penicillin (100 IU per ml) and streptomycin (100 μ g per ml), and 0.2 mg per ml L-glutamine (Gibco-BRL, New York, NY). Cells were seeded with 1×10^6 cells in each culture dish (6 cm in diameter) and incubated at 37°C in 5% CO₂.

UV irradiation source The UVB source was a bank of seven fluorescent sunlamps (FL.20SE.30, Toshiba Medical Supply, Tokyo, Japan) with an emission spectrum of 275–375 nm peaking at 305 nm, emitting mainly in the UVB range but also small amounts of UVA and UVC. The irradiance was 0.3 mW per cm² at a distance of 35 cm measured with a radiometer (UVR-305/365D(II), Toshiba Medical Supply).

Treatment with DDC, TNF- α , or TGF- β_1 After washing HaCaT cells three times with phosphate-buffered saline (PBS), the cells were treated with each reagent. HaCaT cells were incubated with 1 mM DDC dissolved in PBS (pH 7.4) for 1.5 h, 1 ng per ml of TNF- α for 24 h, or 10 ng per ml of TGF- β_1 for 24 h.

UVB irradiation After the treatment with 1 mM DDC, 1 ng per ml of TNF- α , or 10 ng per ml of TGF- β_1 , HaCaT cells were washed three times with PBS. Then, 2 ml of PBS was added to each culture dish. Thereafter, each culture dish of cells was exposed to a single dose of UVB irradiation (10, 20, or 30 mJ per cm²).

Assay of LDH activity LDH leakage from damaged HaCaT cells in the supernatant was measured 8 and 24 h after each dose of UVB irradiation. The LDH activity was spectrophotometrically determined by measuring the reduced nicotinamide adenine dinucleotide (NADH) disappearance rate at 340 nm as a main wavelength during the LDH-catalyzed conversion of pyruvate to lactate according to the Wróblewski-La Due method (Wróblewski and John, 1955). The LDH activity is expressed as U per l at 37°C. Incubation with either 1 mM DDC, 1 ng per ml of TNF- α , or 10 ng per ml of TGF- β_1 for an adequate time did not significantly affect LDH release.

Cell viability Cell viability of HaCaT cells treated with 1 mM DDC, 1 ng per ml of TNF- α , or 10 ng per ml of TGF- β_1 was evaluated by the trypan blue dye exclusion assay 8 and 24 h after each dose of UVB irradiation. We collected both the floating cells in the supernatant and the remaining cells adhering to the dish together using 0.02% ethylenediamine tetraacetic acid (EDTA) and 0.25% trypsin 8 and 24 h after UVB irradiation. Then, both the dead and alive cells were counted using the trypan blue dye exclusion assay. Incubation with 1 mM DDC, 1 ng per ml

of TNF- α , or 10 ng per ml of TGF- β_1 for an adequate time did not significantly affect cell viability.

Measurement of intracellular peroxides by flow cytometry

Intracellular peroxide levels were assessed using an oxidation-sensitive fluorescent probe DCFH-DA. In the presence of a variety of intracellular peroxides, DCFH is oxidized to a highly fluorescent compound, 2',7'-dichlorofluorescein (Bass *et al*, 1983). HaCaT cells, both untreated and treated with 1 mM DDC for 1.5 h, were exposed to a single dose of 10 mJ per cm² UVB; both untreated and treated cells were collected using 0.02% EDTA and 0.25% trypsin 8 h after UVB irradiation. Then, HaCaT cells were incubated with 5 μ M DCFH-DA. The cellular fluorescence intensity, which was directly proportional to levels of intracellular peroxides after 30 min DCFH-DA oxidation, was measured using FACScan (Becton Dickinson, San Jose, CA). For each analysis, 10,000 events were recorded. For image analysis, cells were analyzed for fluorescence intensity using a lysis cell analysis system.

Cu,Zn-SOD and Mn-SOD assays After treatment with DDC (0.01, 0.1, or 1 mM), TNF- α (0.1, 0.5, or 1 ng per ml) or TGF- β_1 (1, 5, 10 ng per ml), cells were collected using 0.02% EDTA and 0.25% trypsin, ruptured by sonication for 30 s using a W-220 sonicator (Heat System-Ultrasonic, NY) at full power, and then centrifuged at 18,000 rpm for 60 min. The supernatant was kept on ice and used for the SOD activity assay and protein determination. SOD activity in HaCaT cells was determined according to the reduction of ferricytochrome c method of McCord and Fridovich (McCord and Fridovich, 1969). A 0.1 ml volume of each supernatant was added to the xanthine-xanthine oxidase O₂⁻-generating system, which consisted of the SOD assay mixture [8.2 mg per ml of ferricytochrome c, 2 mM hypoxanthine, and 50 mM disodium EDTA, 125 mM phosphate buffer (pH 7.8)] in a total volume of 1.8 ml, and 0.1 ml of 0.12 U xanthine oxidase per ml. SOD activity was measured at 25°C. In this system, the formation of O₂⁻ is determined by ferricytochrome c (type III) reduction, and the absorbance was measured using a spectrophotometer (U-3200: Hitachi, Tokyo, Japan) at 550 nm. One unit was defined as the amount of SOD sufficient to inhibit the rate of reduction of ferricytochrome c by 50%, and specific activity was expressed as U per mg protein. To determine Mn-SOD activity, KCN (2 mM) was added to the mixture to inhibit Cu,Zn-SOD activity (Tyler, 1974). Protein concentration was determined with a BCA assay kit (Pierce).

Statistical analysis Statistical significance was assessed by Student's *t* test. Mean differences were considered significant at $p < 0.05$.

RESULTS

Effects of DDC, TNF- α , or TGF- β_1 on total, Cu,Zn-SOD, and Mn-SOD activities in HaCaT cells

Total SOD activities were reduced to 72% ($p < 0.05$) and 48% ($p < 0.01$) of the control level after 1.5 h incubation with 0.1 and 1 mM DDC, respectively. Cu,Zn-SOD activities were reduced to 50% ($p < 0.01$) and 0.3% ($p < 0.01$) of the control level after incubation with 0.1 and 1 mM DDC, respectively, whereas Mn-SOD activities in HaCaT cells were not affected by incubation with DDC (**Fig 1a**). Total SOD activities were increased to 117% ($p < 0.05$) and 147% ($p < 0.01$) of the control level after 24 h incubation with 0.5 and 1 ng per ml of TNF- α , respectively. Mn-SOD activities were increased to 141% ($p < 0.01$) and 196% ($p < 0.01$) of the control level after incubation with 0.5 and 1 ng per ml of TNF- α , respectively, whereas Cu,Zn-SOD activities in HaCaT cells were not affected by incubation with TNF- α (**Fig 1b**). Mn-SOD activities in HaCaT cells were decreased to 78% ($p < 0.05$) of the control level after 24 h incubation with 10 ng per ml of TGF- β_1 , whereas total and Cu,Zn-SOD activities were not affected by incubation with TGF- β_1 (**Fig 1c**).

Increased extracellular leakage of LDH from damaged HaCaT cells treated with DDC following UVB irradiation

UVB-induced cytotoxicity was evaluated by measurement of the extracellular leakage of LDH, a high molecular cytosolic enzyme, and LDH release from damaged keratinocytes increased in a UVB-dose-dependent fashion. The LDH activity released in the supernatant from HaCaT cells treated with 1 mM DDC significantly increased 8 h after 20 and 30 mJ per cm² UVB irradiation ($p < 0.01$) and also 24 h after 10, 20, and 30

mJ per cm² UVB irradiation ($p < 0.01$) compared with untreated groups. Incubation with 1 mM DDC for 8 or 24 h did not

significantly affect LDH release (Fig 2a). On the other hand, no increase of LDH release in the supernatant from HaCaT cells treated with either 1 ng per ml of TNF- α or 10 ng per ml of TGF- β_1 was observed 8 and 24 h after 10, 20, and 30 mJ per cm² UVB irradiation compared with untreated groups (Figs 2b, c).

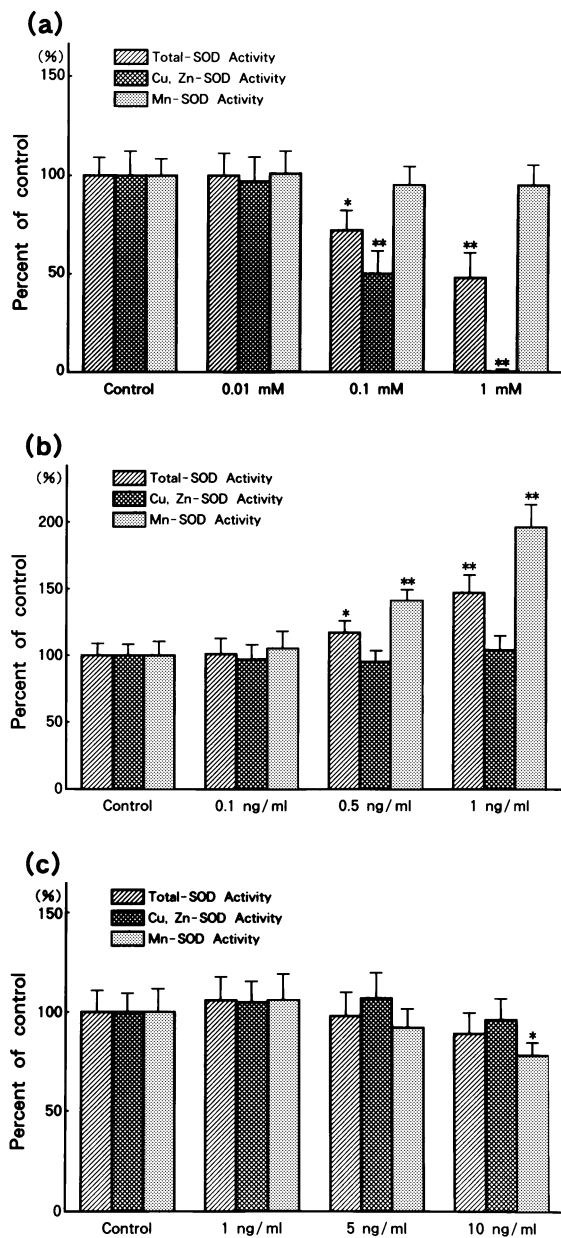


Figure 1. Effects of DDC, TNF- α , or TGF- β_1 on total, Cu,Zn-, and Mn-SOD activities in HaCaT cells. (a) Total, Cu,Zn-SOD, and Mn-SOD activities in HaCaT cells treated with DDC (0.01, 0.1, 1 mM). Total, Cu,Zn-SOD, and Mn-SOD activities are expressed as a percentage of the control value. Control values for total, Cu,Zn-SOD and Mn-SOD activities were 8.0 U per mg protein, 4.2 U per mg protein, and 3.9 U per mg protein, respectively. Data are expressed as mean \pm one standard deviation from the results of six separate experiments. * $p < 0.05$ versus control; ** $p < 0.01$. (b) Total, Cu,Zn-SOD, and Mn-SOD activities in HaCaT cells treated with TNF- α (0.1, 0.5, 1 ng per ml). Total, Cu,Zn-SOD, and Mn-SOD activities are expressed as a percentage of the control value. Control values for total, Cu,Zn-SOD and Mn-SOD activities were 7.6 U per mg protein, 4.0 U per mg protein, and 3.6 U per mg protein, respectively. Data are expressed as mean \pm one standard deviation from the results of six separate experiments. * $p < 0.05$ versus control; ** $p < 0.01$. (c) Total, Cu,Zn-SOD, and Mn-SOD activities in HaCaT cells treated with TGF- β_1 (1, 5, 10 ng per ml). Total, Cu,Zn-SOD, and Mn-SOD activities are expressed as a percentage of the control value. Control values for total, Cu,Zn-SOD, and Mn-SOD activities were 8.1 U per mg protein, 4.3 U per mg protein, and 3.9 U per mg protein, respectively. Data are expressed as mean \pm one standard deviation from the results of six separate experiments. * $p < 0.05$ versus control; ** $p < 0.01$.

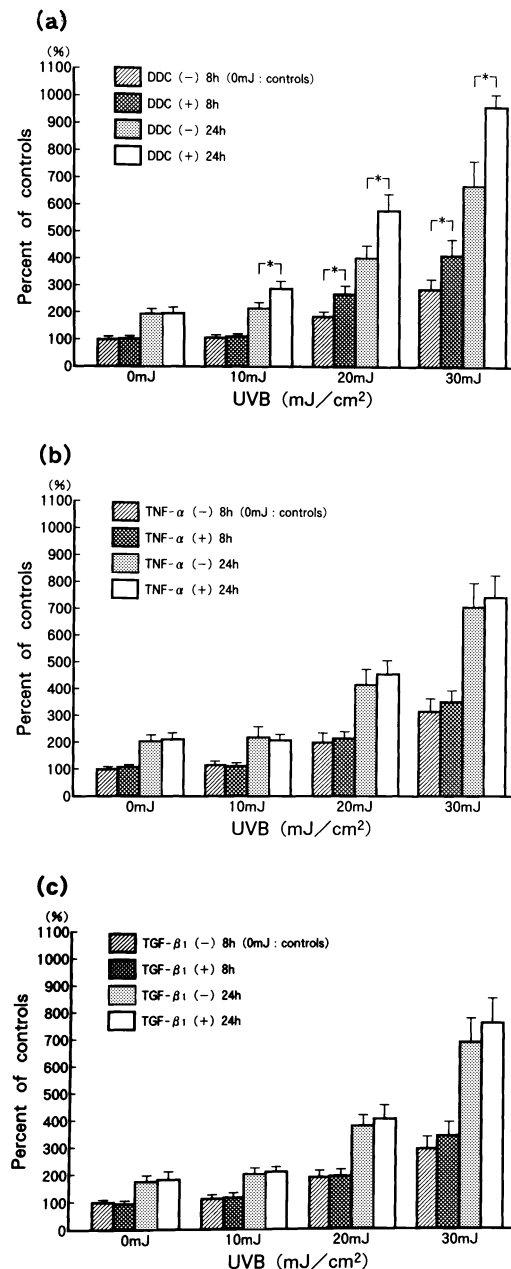


Figure 2. Increased LDH leakage from HaCaT cells treated with DDC, but not TNF- α or TGF- β_1 following UVB irradiation. (a) LDH activities released in the supernatant from HaCaT cells treated with DDC (1 mM) 8 and 24 h after a single exposure to UVB at a dose of 10, 20, or 30 mJ per cm². LDH activities are expressed as a percentage of the control value, 11 U per l. Data are expressed as mean \pm one standard deviation from the results of six separate experiments. * $p < 0.01$ versus control. (b) LDH activities released in the supernatant from HaCaT cells treated with TNF- α (1 ng per ml) 8 and 24 h after a single exposure to UVB at a dose of 10, 20, or 30 mJ per cm². LDH activities are expressed as a percentage of the control value, 28 U per l. Data are expressed as mean \pm one standard deviation from the results of six separate experiments. (c) LDH activities released in the supernatant from HaCaT cells treated with TGF- β_1 (10 ng per ml) 8 and 24 h after a single exposure to UVB at a dose of 10, 20, or 30 mJ per cm². LDH activities are expressed as a percentage of the control value, 34 U per l. Data are expressed as mean \pm one standard deviation from the results of six separate experiments.

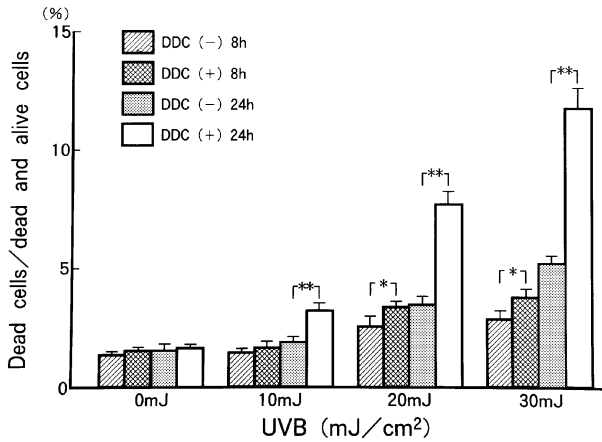


Figure 3. Decreased cell viability of HaCaT cells treated with DDC following UVB irradiation. Cell viability of HaCaT cells treated with DDC (1 mM) 8 and 24 h after a single exposure to UVB at a dose of 10, 20, or 30 mJ per cm². Data are expressed as mean \pm one standard deviation from the results of six separate experiments. * $p < 0.05$ versus control; ** $p < 0.01$.

Decreased cell viability of HaCaT cells treated with DDC following UVB irradiation Cell viability following UVB irradiation was evaluated by the trypan blue dye exclusion assay. Viability of HaCaT cells treated with 1 mM DDC significantly decreased 8 h after 20 and 30 mJ per cm² UVB irradiation ($p < 0.05$) and also 24 h after 10, 20, and 30 mJ per cm² UVB irradiation ($p < 0.01$) compared with untreated groups. Incubation with 1 mM DDC for 8 or 24 h did not significantly affect cell viability (Fig 3). On the other hand, no decrease in viability of HaCaT cells treated with either 1 ng per ml of TNF- α or 10 ng per ml of TGF- β_1 was observed 8 and 24 h after 10, 20, and 30 mJ per cm² UVB irradiation compared with untreated groups (data not shown).

Flow cytometric analysis of intracellular peroxides produced in HaCaT cells treated with DDC following UVB irradiation The production of intracellular peroxides in HaCaT cells untreated or treated with 1 mM DDC following 10 mJ per cm² UVB irradiation was examined by fluorescence-activated cell sorting scan analysis using a peroxide-sensitive dye, DCFH-DA. No increase of intracellular peroxides was observed in the untreated cells 8 h following UVB irradiation (Fig 4a), whereas the levels of intracellular peroxides in the cells treated with DDC significantly increased 8 h following UVB irradiation (Fig 4b).

DISCUSSION

The UVB-induced ROS are generally thought to cause oxidative stress and subsequent photodamage to cellular membrane lipids, proteins, and DNA (Breimer, 1991; Tyrrell, 1995) in human skin, which leads to skin cancer, photoaging (Ananthaswamy and Pierceall, 1990; Miyachi, 1995), and many acute or chronic inflammatory skin disorders (Black, 1987). Because the surface of the skin is always in contact with oxygen and is one of the major targets for UV light, skin requires efficient mechanisms to protect itself from oxidative stress. Antioxidant enzymes such as SOD in the human epidermis are crucial as the outermost barrier against ROS. With regard to the role of SOD against UVB-induced oxidative stress in the epidermis, it has been reported that endogenous SOD levels are decreased after acute UVB irradiation due to scavenging ROS (Hashimoto *et al*, 1991; Punnonen *et al*, 1991). Furthermore, we have recently demonstrated that Cu,Zn-SOD and Mn-SOD change differently following UVB irradiation in the human keratinocyte cell line HaCaT (Sasaki *et al*, 1997), suggesting that endogenous Cu,Zn-SOD and Mn-SOD may play different defensive roles against UVB-induced keratinocyte injury.

In this study, we demonstrated that Cu,Zn-SOD activities in HaCaT cells treated with 1 mM DDC were markedly decreased, whereas Mn-SOD activities were not. Several reports have shown that DDC inactivates Cu,Zn-SOD activities by chelating copper ion, an active center of the enzyme, without affecting other antioxidant enzymes such as Mn-SOD, catalase, and glutathione peroxidase *in vivo* and *in vitro* (Heikila *et al*, 1976; Hiraishi *et al*, 1994). We confirmed that 1 mM DDC does not affect either Mn-SOD (Fig 1), catalase, or glutathione peroxidase activities (data not shown) in HaCaT cells. We used a very low concentration of DDC (1 mM) in this study, because DDC itself possesses strong cytotoxicity.

To evaluate the defensive role of Cu,Zn-SOD against UVB-induced injury of HaCaT cells, we measured LDH release in the supernatant from keratinocytes treated or untreated with DDC 8 and 24 h after UVB irradiation. Extracellular LDH release in the supernatant is well known to be associated with the extent of cell membrane damage in culture cells (Gaboriau *et al*, 1993; Tebbe *et al*, 1997). The LDH release in the culture medium from HaCaT cells treated with 1 mM DDC significantly increased compared with untreated controls 8 and 24 h following UVB irradiation. The viability of HaCaT cells treated with 1 mM DDC significantly decreased compared with untreated controls 8 and 24 h following UVB irradiation. These results suggest that Cu,Zn-SOD may play a

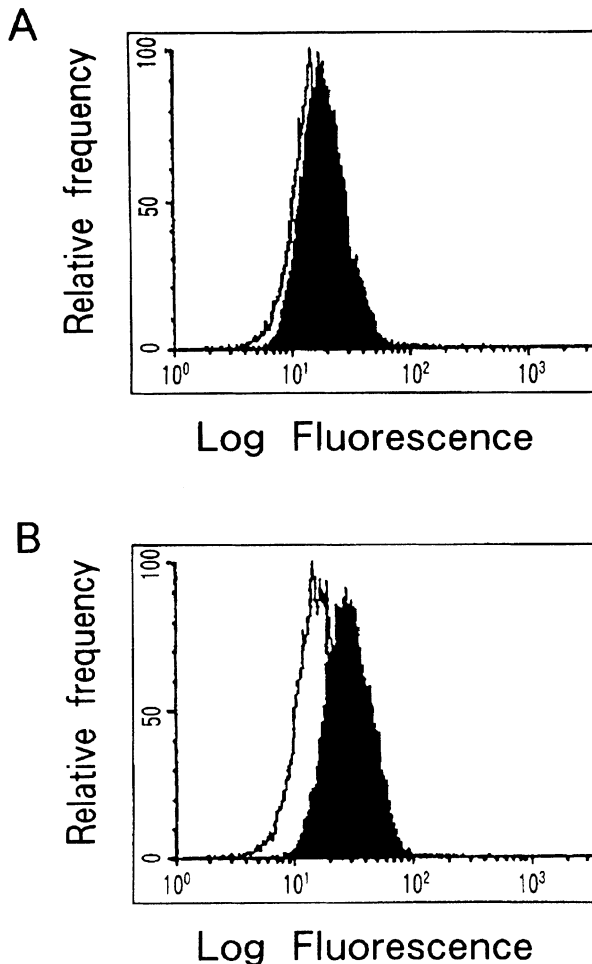


Figure 4. Increased production of intracellular peroxides in HaCaT cells treated with DDC following UVB irradiation. Flow cytometric analysis of intracellular peroxides in HaCaT cells untreated (A) or treated (B) with 1 mM DDC. Both untreated and treated cells were irradiated with 10 mJ per cm² UVB (black area) or were unirradiated (white area). Relative peroxide concentrations in the cells were quantitated by flow cytometry using a peroxide-sensitive dye, DCFH-DA, 8 h following UVB irradiation.

role as an early phase defense mechanism against UVB-induced cytotoxicity in HaCaT cells.

Next, we demonstrated that Mn-SOD activities but not Cu,Zn-SOD activities in HaCaT cells were markedly enhanced after the treatment with 1 ng per ml of TNF- α . In a previous study we have shown that 1 ng per ml of TNF- α markedly enhances Mn-SOD activities in HaCaT cells (Sasaki *et al.*, 1997). In this study we demonstrated that the treatment with 10 ng per ml of TGF- β_1 significantly inhibits Mn-SOD activities in HaCaT cells, whereas Cu,Zn-SOD activities are not affected by TGF- β_1 . TGF- β_1 , which plays a central role in negative regulation of cell growth, has been reported to suppress antioxidant enzyme gene expression, mainly Mn-SOD and glutathione-S-transferase, in rat hepatocytes (Kayanoki *et al.*, 1994). We here report that TGF- β_1 suppresses Mn-SOD activities in human keratinocytes.

To evaluate the defensive role of Mn-SOD against UVB-induced injury of HaCaT cells, we measured LDH release in the supernatant from damaged keratinocytes treated or untreated with 1 ng per ml of TNF- α after UVB irradiation. The treatment with 1 ng per ml of TNF- α had no effect on the LDH release in the culture medium from HaCaT cells 8 and 24 h following UVB irradiation. We also examined the effect of TGF- β_1 . The treatment with 10 ng per ml of TGF- β_1 had no effect on the LDH release in the culture medium from HaCaT cells 8 and 24 h following UVB irradiation. Moreover, we also examined the viability of HaCaT cells treated with TNF- α or TGF- β_1 after UVB irradiation. The treatment with either 1 ng per ml of TNF- α or 10 ng per ml of TGF- β_1 had no significant effect on the viability of HaCaT cells 8 and 24 h following UVB irradiation compared with untreated groups (data not shown). These results seem to indicate that Mn-SOD does not participate in an early phase defense mechanism against UVB-induced cytotoxicity. Although there have been no reports concerning the role of Mn-SOD against UVB-induced oxidative stress in the epidermis, Mn-SOD may be related to a chronic phase defense mechanism against UVB-induced cutaneous disorders.

To confirm a protective role of Cu,Zn-SOD against UVB-induced early phase cytotoxicity in keratinocytes, we examined the production of intracellular peroxides following UVB irradiation using flow cytometry. Flow cytometric analysis of peroxides 8 h after UVB irradiation showed that the production of intracellular peroxides was increased in the HaCaT cells treated with 1 mM DDC compared with nonirradiated controls. On the other hand, there were no significant differences between untreated HaCaT cells after UVB irradiation and nonirradiated controls. The results of flow cytometric analysis 24 h after UVB irradiation were similar to those 8 h after irradiation (data not shown). It is generally accepted that UV-induced ROS causes lipid peroxidation of plasma membrane, which is well known to be associated with the extent of cellular damage *in vitro* (Girotti, 1990; Morlière *et al.*, 1990). The induction of lipid peroxidation following UVB or UVA exposure in human keratinocytes or fibroblasts has been documented previously (Iizawa *et al.*, 1994; Morlière *et al.*, 1995). Our results suggest that Cu,Zn-SOD may play an early phase protective role against UVB-induced lipid peroxidation.

In this study, increased cytotoxicity following UVB irradiation was observed in the HaCaT cells whose Cu,Zn-SOD levels were inactivated by DDC, as determined by LDH leakage in the supernatant and cell viability 8 and 24 h after UVB irradiation and also by flow cytometric analysis of intracellular peroxides 8 h after UVB irradiation. Treatment with 1 mM DDC had no effect on either LDH leakage, cell viability (Figs 2a, 3), or intracellular peroxide production (data not shown). On the other hand, no significant differences were observed in HaCaT cells whose Mn-SOD was enhanced or inhibited with cytokines. Our results suggest that Cu,Zn-SOD plays a primary protective role against UVB-induced acute phase keratinocyte injury, presumably due to more widespread and predominant distribution of endogenous Cu,Zn-SOD than Mn-SOD in cells.

It remains unclear whether Mn-SOD does not actually participate in an acute phase defense mechanism against UVB-induced injury of HaCaT cells, although Mn-SOD is decreased following UVB irradiation. It was difficult to evaluate the possible protective role of Mn-SOD against acute phase UVB-induced keratinocyte injury in this study, because there is no reagent that perfectly inhibits Mn-SOD. In fact, we demonstrated that TGF- β_1 at a concentration of 10 ng per ml, which is known to suppress Mn-SOD in rat hepatocytes, suppressed only 22% of Mn-SOD activities, whereas DDC at a concentration of 1 mM perfectly inactivated Cu,Zn-SOD activities in HaCaT cells. We have also examined the protective role of Cu,Zn-SOD or Mn-SOD against UVA-induced cytotoxicity in HaCaT cells. We measured LDH release from DDC or TGF- β_1 treated cells following 1, 5, or 10 J per cm² UVA irradiation. The results showed that LDH release from HaCaT cells treated with TGF- β_1 whose Mn-SOD activity was suppressed significantly increased after UVA irradiation compared with control groups. On the other hand, no significant increase of LDH leakage was observed in the supernatant of the cells whose Cu,Zn-SOD activity was inactivated by DDC after UVA irradiation (data not shown). These results suggest that Mn-SOD may take part in the protection against UVA-induced injury of the human keratinocyte. Further experiments using other methods are required to clarify these points.

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