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UVA-Induced Immune Suppression Through an Oxidative Pathway

Ichiro Iwai, Masato Hatao, Masako Naganuma, Yoshimaru Kumano, and Masamitsu Ichihashi*

Shiseido Research Center, Yokohama, Japan; *Department of Dermatology, School of Medicine, Kobe University, Hyogo, Japan

Although ultraviolet B (UVB) irradiation induces local immune or systemic immune suppression, depending on the dose, the immune suppression by ultraviolet A (UVA) has not been fully investigated. In this study, we investigated the effect of UVA on the immune response *in vitro* and *in vivo*. The effect of UVA on the antigen-presenting function of epidermal cells was measured in terms of antigen-specific T cell proliferation. A murine epidermal cell suspension was exposed to UVA *in vitro*, pulsed with trinitrobenzenesulfonic acid, and cultured with T cells prepared from syngeneic mice previously sensitized with trinitrochlorobenzene. UVA (5–20 J per cm^2) suppressed the antigen-presenting function of epidermal cells in a dose-dependent manner, accompanied with suppression of the expression of costimulatory molecules on Langerhans cells. In order to investigate the effect of an antioxidant on the immune suppression, an epidermal cell suspension was irradiated with UVA in the presence or absence of glutathione. The suppressions of antigen-presenting function and ICAM-1 expression were significantly prevented by glutathione in a dose-dependent manner. Further, the effect of UVA on the immune response at the induction phase of contact hypersensitivity was evaluated in terms of lymph node cell proliferation *ex vivo*. UVA irradiation suppressed the endogenous proliferation of lymph node cells in trinitrochlorobenzene-painted mice, and this suppression was significantly reversed by the application of glutathione to the skin during irradiation. These results suggest that UVA-induced immune suppression may be mediated by reactive oxygen species, at least in part. ***Key words: antigen presentation/glutathione/Langerhans cells/reactive oxygen species. J Invest Dermatol 112:19–24, 1999***

The exposure of skin to ultraviolet B (UVB) radiation leads to modulation of various biologic processes throughout the whole body. UVB-induced immune suppression is well documented, e.g., in studies on contact hypersensitivity (Noonan *et al*, 1981), delayed-type hypersensitivity (Ullrich *et al*, 1986), and tumor rejection (Kripke, 1984). Acute UVB (100 mJ per cm^2) (Kripke *et al*, 1992) exposure or chronic simulated solar UV (85 J per cm^2) (Ho *et al*, 1991) exposure for 4 wk induced immune suppression *in vivo*. UVB (2.5–20 mJ per cm^2) irradiation *in vitro* induced suppression of Langerhans cell function (Stingl *et al*, 1981; Simon *et al*, 1991; Tang and Udey, 1991; Rattis *et al*, 1995). In addition, susceptibility to UVB-induced immune suppression supposedly has a close relation to the development of skin cancers in humans (Yoshikawa *et al*, 1990). The mechanism of the immune suppression induced by UVB is thought to involve Langerhans cells, which are major antigen-presenting cells that trap antigens in the skin and trigger sequential immunologic events (Toews *et al*, 1980). There have been many studies of the direct effect of UVB (Simon *et al*, 1990, 1991) or the indirect effect of soluble factors secreted from UVB-irradiated keratinocytes (Rivas and Ullrich, 1992; Enk *et al*, 1993) on Langerhans cells. Whereas commercially available sunscreens with a high SPF value can protect the skin from UVB, they are not always protective against UV-induced immune suppression (Ho *et al*, 1992; van Praag *et al*, 1991; Wolf *et al*, 1993). Thus, the effect of ultraviolet A (UVA) that passes through sunscreens on the immune responses has attracted interest. It is known that UVA induces DNA damage (Roza *et al*, 1989), DNA-protein cross-links (Peak *et al*, 1985), and membrane damage (Black, 1987; Tyrrell and Keyse, 1990; Shindo *et al*, 1994); however, the effect of UVA on the immune system is still controversial. Under some experimental conditions, UVA irradiation decreases the number of Langerhans cells in the skin (Alcalay *et al*, 1989; Lavker *et al*, 1995; Grabbe *et al*, 1996). UVA (2.5–20 J per cm^2) irradiation *in vitro* induces suppression of Langerhans cell function (Clement-Lacroix *et al*, 1996). There is another study reporting UVA (46 J per cm^2)-induced immune suppression *in vivo* (Bestak and Halliday, 1996), but on the other hand it has been found that UVA has no effect on contact hypersensitivity (Laihia and Jansen, 1994).

Therefore, in order to confirm that UVA can induce immune suppression, we investigated the effect of UVA on the proliferation of T cells as a result of antigen-presenting ability of epidermal cells (Stingl *et al*, 1978; Braathen and Thorsby, 1980). We also measured the expression of cell surface molecules ICAM-1, B7–1, and B7–2 on UVA-irradiated Langerhans cell by flow cytometric analysis, because they are known to play an important role in antigen presentation to T cells (Dang *et al*, 1990; Tang and Udey, 1991; Gaspari *et al*, 1993; Symington *et al*, 1993). Furthermore, UVA is known to produce reactive oxygen species (Shindo *et al*, 1993, 1994). To determine whether immune suppression induced by UVA was due to reactive oxygen species, we investigated the effect of glutathione on the antigen-presenting function of epidermal

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Reprint requests to: Dr. Masato Hatao, Address: 1050 Nippa-cho, Kohoku-ku, Yokohama 223, Japan.

Abbreviations: TNBS, trinitrobenzenesulfonic acid; TNCB, trinitrochlorobenzene.

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cells and on *ex vivo* lymph node cell proliferation (Kimber *et al*, 1993; Hatao *et al*, 1994).

MATERIALS AND METHODS

Mice Specific-pathogen-free female C3H/HeN mice were obtained from the Shizuoka Laboratory Animal Center (Shizuoka, Japan) and were used at 8–12 wk of age. The following experiments conform to the guide for the care and use of laboratory in NIH.

Preparation of epidermal cells Epidermal cells were prepared as described before (Stingl *et al*, 1981). Briefly, ears from mice were incubated in a phosphate-buffered saline (GIBCO, Rockville, MD) solution containing 0.5% trypsin (Sigma, St. Louis, MO) for 30 min at 37°C. Epidermal sheets were removed and teased to produce a single cell suspension of epidermal cells in a 0.05% deoxyribonuclease 1 (Sigma)-RPMI1640 (GIBCO) solution supplemented with 10% heat-inactivated fetal bovine serum (GIBCO).

UVA irradiation of epidermal cells Epidermal cells were suspended in RPMI/10% heat-inactivated fetal bovine serum, in a polystyrene dish (2×10^6 cells in 3 ml) and irradiated with UVA through a UVB cut filter (Schott WG335, Shott Glas, Mainz, Germany) in the presence or absence of glutathione (Sigma). The light source was a bank of six FL20S-BLB fluorescent tubes with an emission spectrum of 310–410 nm and a peak at 350 nm (Toshiba, Tokyo, Japan). The irradiance of UVA was about 1.5 mW per cm^2 for *in vitro* irradiation and 5 mW per cm^2 for *in vivo* irradiation. The UV intensity was measured with a UVR-3036S Radiometer (Torex, Tokyo, Japan). Sham-treated cell populations served as the control. Cell viability was assessed by trypan blue exclusion.

Antigen pulsing Epidermal cells suspended in RPMI supplemented with 10% heat-inactivated fetal bovine serum were incubated for 30 min at 37°C with 1 mg per ml of 2,4,6-trinitrobenzenesulfonic acid (TNBS; Sigma). After antigen pulsing, cells were washed three times and resuspended in RPMI/10% heat-inactivated fetal bovine serum at a concentration of 1×10^6 cells per ml.

Preparation of T cells Mice were sensitized on their ears with 25 μl of 3% trinitrochlorobenzene (TNCB; Tokyo Kasei, Tokyo, Japan) solution in acetone:olive oil (4:1 vol/vol) for two consecutive days. Four days after the last TNCB treatment, regional lymph nodes (auricular lymph nodes) were excised, and disrupted, and the resulting cell suspension was collected. Cells were then passed over a nylon fiber column (Wako, Osaka, Japan). Non-adherent, eluted cells were resuspended at a concentration of 2×10^6 cells per ml. Prepared cells consisted of more than 95% $CD4^+$ or $CD8^+$ T cells, determined by flow cytometric analysis.

Evaluation of the antigen-presenting function of epidermal cells Sensitized T cells (2×10^5 per 100 μl) and antigen-primed epidermal cells (1×10^5 per 100 μl) were mixed and cultured in a 96 well plate for 72 h as described before (Stingl *et al*, 1981). Eighteen hours before harvesting, 0.5 μCi of [^{3}H]thymidine (Daiichikagakuyakuhin, Tokyo, Japan) was added to each well. Radioactivity of [^{3}H]thymidine incorporated by proliferating cells was measured by a liquid scintillation counter.

Flow cytometric analysis of costimulatory molecules on Langerhans cells After UVA exposure, the cells were collected, washed, and cultured for 24 h. Immediately after and at 24 h after UVA irradiation, the cell-surface molecules were immunostained in PBS supplemented with 1% fetal bovine serum and 0.2% NaN_3 (Sigma). Fluorescein isothiocyanate-conjugated anti-mouse I-A^k monoclonal antibody, phycoerythrin-conjugated anti-mouse CD54 (ICAM-1) monoclonal antibody, biotin-conjugated hamster anti-mouse CD80 (B7–1) monoclonal antibody, biotin-conjugated rat anti-mouse CD86 (B7–2) monoclonal antibody and phycoerythrin-conjugated streptavidin were purchased from Pharmingen (San Diego, CA). Stained cells were washed three times, resuspended, and analyzed by a flow cytometer (EPICS XL, Coulter, Hialeah, FL). Langerhans cells were identified as IA^k positive cell subset, and the expression of costimulatory molecules were evaluated by double staining.

Lymph node cell proliferation assay The effect of UVA on the immune system *in vivo* was evaluated by means of a lymph node cell proliferation assay modified from a reported *ex vivo* local lymph node assay (Hatao *et al*, 1994). Briefly, the dorsal hair of the mice was depilated by commercially available hair-remover (Shiseido, Osaka, Japan) and the skin was irradiated with UVA (130 J per cm^2). During the UVA irradiation, 15 μl of 3% glutathione solution in 50% ethanol was topically applied every 2 h. Twenty-four hours after the irradiation, a 3% solution of TNCB was painted on the irradiated sites. Inguinal lymph nodes were excised 3 d after sensitization and disrupted. The resulting cells were washed, resuspended in RPMI/10% heat-inactivated fetal bovine serum at a concentration of 5×10^6 cells per ml, and cultured in a 96 well microtiter plate for 24 h. Eighteen hours before harvesting, 0.5 μCi of [^{3}H]thymidine was added to each well. Endogenous proliferation of the cells was evaluated in terms of the uptake of [^{3}H]thymidine.

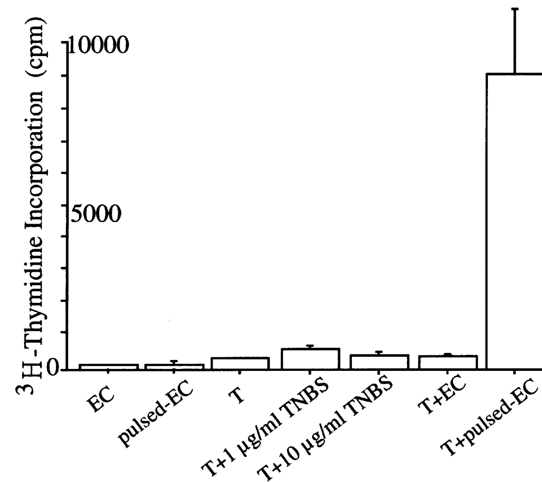

Figure 1. Antigen-pulsed epidermal cells induced proliferation of T cells enriched from lymph node cell suspensions. T cell proliferation was evaluated by [^{3}H]thymidine incorporation. EC, intact epidermal cells; pulsed-EC, pulsed epidermal cells; T, T cell-enriched fraction of lymph node cells from TNCB-sensitized mice; T + TNBS, T cell-enriched fraction of lymph node cells from TNCB-sensitized mice with added antigen; T + EC, T cell-enriched fraction with epidermal cells; T + pulsed-EC, T cell-enriched fraction with pulsed epidermal cells. Data shown are from one representative experiment out of two independent experiments that gave similar results (mean ± SD of triplicate cultures).

Statistical evaluation of results The statistical significance of differences in the means of each data was calculated with Student's t test. Mean differences were considered to be significant at $p < 0.05$.

RESULTS

Antigen-pulsed epidermal cells induce proliferation of T cells enriched from the suspensions of lymph node cells Intact epidermal cells or pulsed epidermal cells showed a very low [^{3}H]thymidine incorporation. T cell-enriched fraction of lymph node cells from TNCB-sensitized mice with or without TNBS added in the medium did not show proliferation either; however, T cell-enriched fraction cocultured with pulsed epidermal cells showed marked proliferation (**Fig 1**).

UVA exposure inhibits antigen-presenting function of epidermal cells The proliferation of T cells caused from the antigen presentation by TNBS-pulsed epidermal cells was apparently inhibited by UVA irradiation (0–20 J per cm^2) in a dose-dependent manner (**Fig 2**). The cytotoxicity was not observed when epidermal cells were irradiated with 2.5–5 J UVA per cm^2; however, 7.5–10 J UVA per cm^2 decreased viability of epidermal cells (**Fig 3**). Similar suppression was observed when epidermal cells were exposed to UVA either in Hank's balanced salt solution or in RPMI/10% fetal bovine serum without phenol red, suggesting that the major part of the suppression was not due to the phototoxic effect of phenol red under this condition (data not shown).

UVA exposure inhibits upregulation of accessory molecules on Ia-positive Langerhans cell The expression of ICAM-1 on Ia-positive Langerhans cells was increased after 24 h of cell culture, as reported previously (Tang and Udey, 1991). In this series of experiments, UVA irradiation clearly inhibited this upregulation of

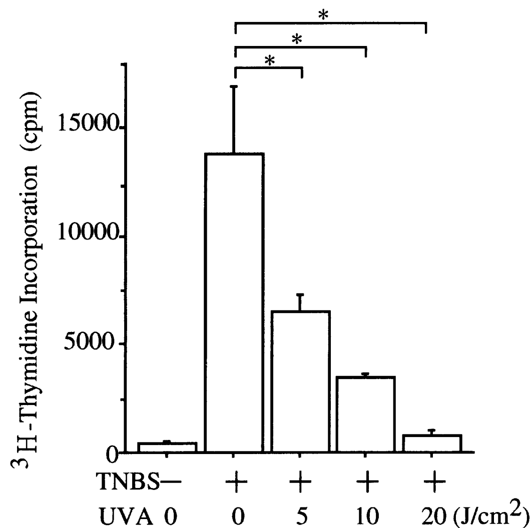

Figure 2. Dose-dependent suppression of antigen-presenting capacity of epidermal cells by UVA irradiation. UVA (0–20 J per cm^2)-irradiated murine epidermal cells were pulsed with TNBS and were cultured with T cells prepared from syngeneic mice sensitized with TNBC. The antigen-presenting function of Langerhans cell is expressed in terms of radioactivity of [^{3}H]thymidine incorporated by proliferating T cells. Data shown are from one representative experiment out of two independent experiments that gave similar results (mean ± SD of triplicate cultures). The asterisk indicates a statistically significant difference ($p < 0.05$) between the UVA-irradiated cells and sham-irradiated cells.

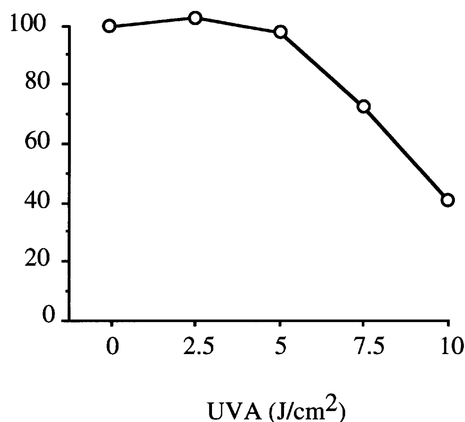

Figure 3. Cell survival of UVA-irradiated epidermal cells. Twenty-four hours after UVA (2.5–10 J per cm^2) irradiation, the viability of epidermal cells was assessed by trypan blue exclusion. Survival (%) was determined by use of the following formula: (viability of UVA-irradiated epidermal cells) ÷ (viability of sham-irradiated epidermal cells) × 100.

ICAM-1 in a dose-dependent manner (**Fig 4*a***). A similar inhibitory effect was observed for other costimulatory molecules, B7–1 and B7–2 (**Fig 4*b***, ***c***).

Supernatants harvested from UVA-irradiated keratinocyte had no effect on the proliferation of T cells In order to confirm that the suppression is due to the direct effect of UVA irradiation, supernatants from keratinocyte culture were added to the coculture media. Supernatants harvested from keratinocyte culture 24, 48, 72 h after UVA irradiation had no effect on the proliferation of T cells (**Fig 5**). Supernatants harvested from keratinocyte culture 24 h after irradiation also had no effect on ICAM-1 expression of Langerhans cells either (data not shown).

Protective effect of glutathione against UVA-induced suppression of the antigen-presenting function of epidermal cells The effects of glutathione on UVA-induced suppression of

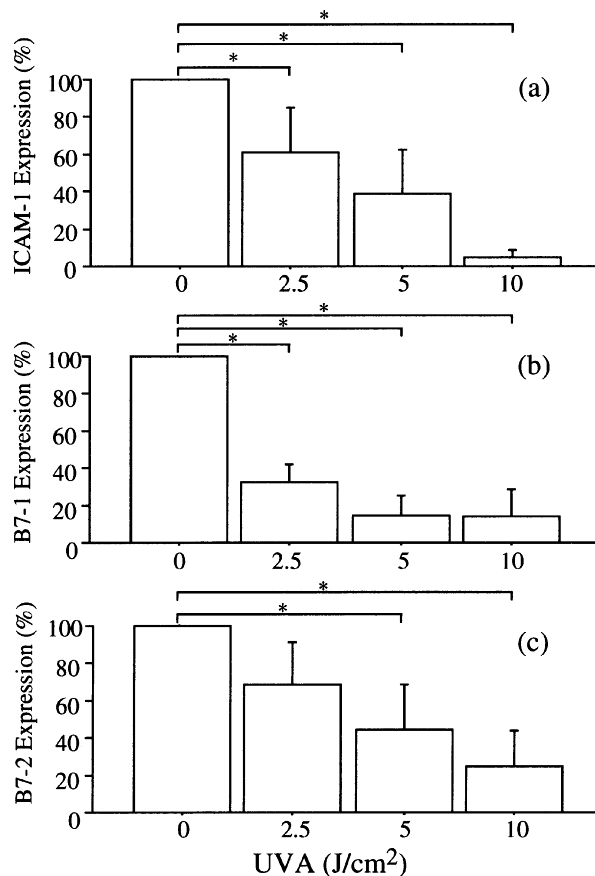

Figure 4. Dose-dependent suppression of upregulation of the ICAM-1, B7–1, and B7–2 expression on Langerhans cell by UVA irradiation. Freshly isolated and cultured epidermal cells were incubated with fluorescein isothiocyanate-conjugated anti-Ia antibody and phycoerythrin-conjugated anti-CD54 antibody for analysis of the ICAM-1 expression (*a*), and with fluorescein isothiocyanate-conjugated anti-Ia antibody and biotin-conjugated anti-CD80 antibody for analysis of B7–1 (*b*) or biotin-conjugated anti-CD86 antibody for analysis of B7–2 (*c*), followed by phycoerythrin-conjugated streptavidin. Expression (%) of these surface molecules was calculated by use of the following formula: (fluorescence intensity of UV-irradiated cultured Langerhans cells − fluorescence intensity of fLangerhans cells) ÷ (fluorescence intensity of sham-irradiated cultured Langerhans cells − fluorescence intensity of fLangerhans cells) × 100. Data were expressed as the mean ± SD of three independent experiments. The asterisk indicates a statistically significant difference ($p < 0.05$) between the UVA-irradiated cells and sham-irradiated cells.

antigen-presenting function were investigated. The augmented [^{3}H]thymidine incorporation by T cells was suppressed by 5 J per cm^2 of UVA. This suppression was significantly mitigated by 3 mM glutathione added to the cell-culture media during irradiation (**Fig 6**). One millimolar glutathione showed a less marked, but still significant, mitigating effect, whereas 0.3 mM glutathione had no effect. The antigen-presenting function of sham-irradiated cells was not affected by these doses of glutathione. The same results were also obtained when epidermal cells were exposed in the medium without phenol red (data not shown).

Protective effect of glutathione against UVA-induced suppression of the surface ICAM-1 expression on Langerhans cells In order to elucidate the mechanism of the mitigating effect of glutathione on the antigen-presenting function, the effect of glutathione on the UVA-induced suppression of surface ICAM-1 expression was investigated by flow cytometry. The dose-dependent suppression of ICAM-1 expression by UVA was significantly mitigated by glutathione (**Fig 7**). Three millimolar glutathione almost blocked the suppression induced by 2.5 J per cm^2 of UVA. Furthermore, the suppression of ICAM-1 expression induced by

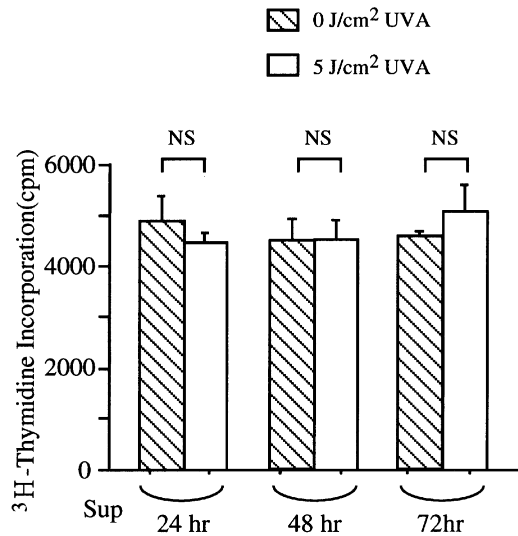

Figure 5. Effect of supernatants from UVA-irradiated epidermal cells on Langerhans cell function. Intact epidermal cells and T cells were cocultured in the supernatants harvested from UVA-irradiated or unirradiated epidermal cells that had been cultured for 1–3 d after irradiation. Data shown are from one representative experiment out of two independent experiments that gave similar results (mean ± SD of triplicate cultures).

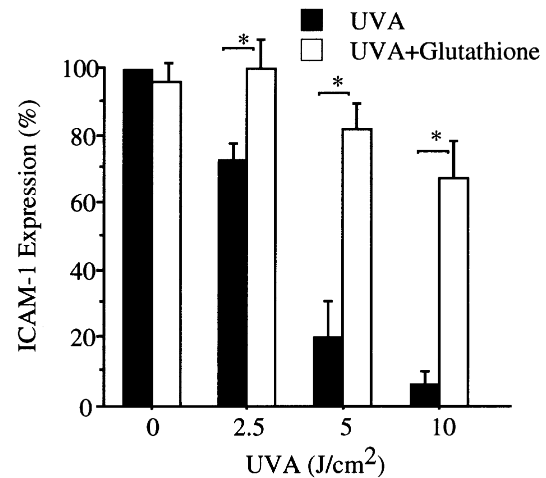

Figure 7. Protective effect of glutathione against UVA (2.5–10 J per cm^2)-induced suppression of ICAM-1 expression. Epidermal cells were irradiated with UVA (2.5–10 J per cm^2) in the presence or absence of 3 mM glutathione. The ICAM-1 expression (%) was calculated as described in the legend to **Fig 2**. Data were expressed as the mean ± SD of three independent experiments. The asterisk indicates a statistically significant difference ($p < 0.05$) between the glutathione treated cells and untreated cells.

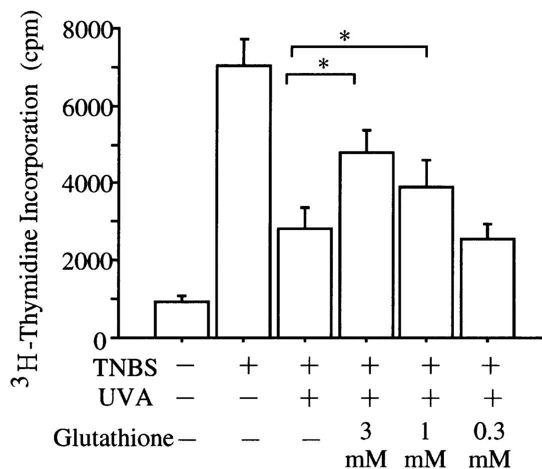

Figure 6. Protective effect of glutathione against UVA-induced suppression of Langerhans cell function. Epidermal cells suspended in RPMI/10% fetal bovine serum were irradiated with UVA (5 J per cm^2) in the presence (0.3–3 mM) or absence of glutathione. Data are from one representative experiment out of two independent experiments (mean ± SD of triplicate cultures). The asterisk indicates a statistically significant difference ($p < 0.05$) between the glutathione treated cells and untreated cells.

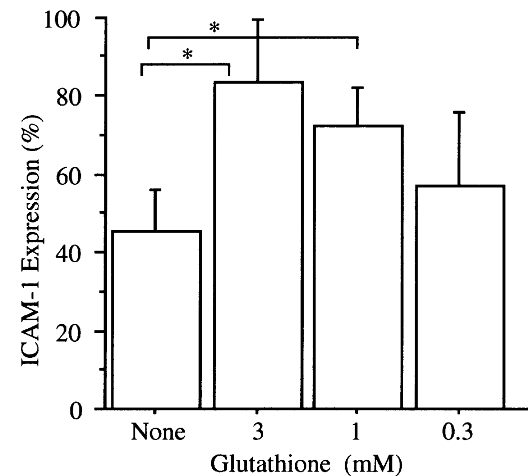

Figure 8. Dose-dependent protective effect of glutathione against UVA-induced suppression of ICAM-1 expression. Epidermal cells were irradiated with UVA (5 J per cm^2) in the presence (0.3–3 mM) or absence of glutathione. [The ICAM-1 expression (%) was calculated as described in the legend to **Fig 4**.] Data are expressed as the mean ± SD of three independent experiments. The asterisk indicates a statistically significant difference ($p < 0.05$) between the glutathione treated and untreated cells.

5 J per cm^2 of UVA was reversed by glutathione in a dose-dependent manner, though 0.3 mM glutathione did not have a significant effect (**Fig 8**).

Evaluation of immune suppression induced by *in vivo* UVA irradiation At the induction phase of contact hypersensitivity, T cells proliferate at the regional lymph node upon receiving an immunogenic signal from antigen-presenting cells. Thus, endogenous lymph node cell proliferation has been used for the assessment of allergenicity of chemicals (Hatao *et al*, 1994). We employed this method to investigate the *in vivo* effect of UVA on the immune system. *In vivo* irradiation with UVA at 130 J per cm^2 significantly decreased the endogenous proliferation of inguinal lymph node cells prepared 24 h after irradiation. The topical application of glutathione during the irradiation showed a protective effect (**Fig 9**).

DISCUSSION

Many studies have shown that UVB suppresses the immune function. Because Langerhans cell plays an important role in the cutaneous immune response, the effect of UVB on Langerhans cell has been extensively investigated (Toews *et al*, 1980; Simon *et al*, 1990, 1991; Enk *et al*, 1993); however, there have been only a few studies regarding the effect of UVA on the immune response (Beasley *et al*, 1996; Bestak and Halliday, 1996). Clement-Lacroix *et al* reported the UVA-induced suppression of allogeneic MELR and observed a protective effect of vitamin E in human epidermal cells *in vitro* (Clement-Lacroix *et al*, 1996). Bestak *et al* suggested that protection against immune suppression by sunscreens is not related to the sun protection factor, but rather to the sunscreens having a broad absorption spectrum (Bestak *et al*, 1995). In this study, epidermal cells from mice were exposed to UVA *in vitro* and

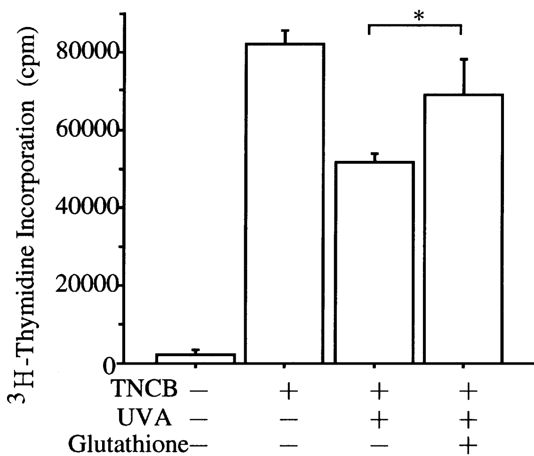

Figure 9. Protective effect of glutathione against the suppression of endogenous proliferation of lymph node cells induced by *in vivo* UVA irradiation. The back of mice was exposed to 130 J per cm^2 UVA. During irradiation, 15 μl of 3% glutathione solution or vehicle was topically applied every 2 h. TNCB was applied to the irradiated sites on the next day. Radioactivity of [^{3}H]thymidine incorporated by regional lymph node cells was measured 3 d after sensitization. Data are from one representative experiment out of three independent experiments that gave similar results (mean ± SD of triplicate cultures). The asterisk indicates a statistically significant difference ($p < 0.05$) between the glutathione treated cells and untreated cells.

the effects of UVA on antigen-presenting function of epidermal cells and expression of costimulatory molecules on Langerhans cell were investigated. We showed that UVA irradiation dose dependently decreased the ability of epidermal cells to present antigen to T cells directly. This phenomenon was accompanied with suppression of the expression of accessory molecules (ICAM-1, B7–1, B7–2). Thus, it is suggested that the suppression of antigen-presenting function may be due to the suppression of accessory molecule expression. We also showed that 130 J per cm^2 of UVA irradiation *in vivo* decreased the endogenous proliferation of lymph node cells. These *in vitro* and *in vivo* data suggest that UVA suppresses the immune response by modulating Langerhans cell function.

As UVA is known to produce reactive oxygen species, these species may play a role in the phenomena described here. Cells have several natural defense systems against oxidative stress, but they cannot always prevent oxidative stress induced by UVA irradiation (Shindo *et al*, 1993, 1994). Among the endogenous free radical scavengers, glutathione plays a major role in preventing UVA-induced oxidative stress (Tyrrell and Pidoux, 1986, 1988). Studies with mouse skin showed that cutaneous glutathione rapidly decreased following UVA irradiation (Wheeler *et al*, 1986; Connor and Wheeler, 1987). We evaluated the effect of exogenous glutathione on UVA-induced immune suppression to establish whether reactive oxygen species are involved.

Suppression of the antigen-presenting function of epidermal cells and the ICAM-1 expression on Langerhans cell induced by UVA (5 J per cm^2) was clearly prevented by the addition of glutathione, in a dose-dependent manner. Glutathione was included in the culture media only during UVA irradiation, and has no absorption in the UVA range. Also, 5 J per cm^2 of UVA was not cytotoxic to epidermal cells. These results support the participation of reactive oxygen species in the suppression of the antigen-presenting function of Langerhans cell.

Previous studies have shown that UV induces cellular damage at the plasma membrane, and that subsequent signal transduction activates NF-κB or AP-1 (Devary *et al*, 1992, 1993). N-Acetyl-cysteine, a precursor of glutathione, added to the cellular medium was transported into the cells and increased the intracellular level of glutathione (Meister, 1991). The increased glutathione protected the plasma membrane from UV-induced damage (Devary *et al*, 1993). In general, intracellular glutathione is exported from the cytosol to the surrounding medium (Bannai and Tsukeda, 1979), but extracellular glutathione is not transported into the cell, except by an Na^+-dependent glutathione transport system found in intestine (Linder *et al*, 1984) and kidney (Lash and Jones, 1984). In our study, most of the glutathione added to the medium might not have been taken up by epidermal cells, but presumably protected the plasma membrane externally against reactive oxygen species. This hypothesis remains to be confirmed.

In addition to these *in vitro* experiments, we demonstrated that glutathione applied to the skin partially prevented the UVA-induced immune suppression *in vivo*. We believe this preventive effect of glutathione should be based on an antioxidative mechanism either at the surface of the skin or inside the skin, because glutathione does not have absorption at UVA range.

Furthermore, in a previous study, vitamin E was found to be effective in reversing the UVA-induced suppression of allogeneic MELR (Clement-Lacroix *et al*, 1996). As vitamin E is also an antioxidant, it seems likely that antioxidants may generally be effective to prevent UVA-induced immune suppression. Thus, we consider that UVA impairs the immune system at least partially via an oxidative pathway. If this is so, the application of sunscreens containing antioxidants, such as glutathione, should be effective in preventing the immune suppression induced by UV.

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