Cutaneous Metallothionein Induction by Ultraviolet B Irradiation in Interleukin-6 Null Mice

Noriko Nishimura, Vivienne E. Reeve,* Hisao Nishimura,† Masahiko Satoh, and Chiharu Tohyama

Environmental Health Sciences Division, National Institute for Environmental Studies (NIES), Tsukuba, Japan; *Department of Veterinary Anatomy and Pathology, University of Sydney, Sydney, Australia; †Department of Human Sciences, Aichi Mizuho University, Aichi, Japan

The mediators of cutaneous metallothionein induction by ultraviolet radiation have not been defined. In this study we sought to identify cytokines that might be involved. We examined the role of interleukin-6, using the IL-6 null (IL-6–/–) mouse, which has been observed to be highly sensitive to ultraviolet radiation damage. Whereas cutaneous metallothionein concentration, measured by radioimmunoassay, began to rise in wild-type (IL-6+/+) mice by 12 h after ultraviolet irradiation, there was a significant delay in the IL-6–/– mice until 48 h after UV irradiation. Immunohistologically, metallothionein appeared in IL-6+/+ mice at 24 h in dermal fibroblasts, and then by 48 h in epidermal basal keratinocytes, with intensity increasing until 72 h, and was coincident with proliferating cell nuclear antigen-positive staining. Corresponding metallothionein expression in IL-6–/– mouse skin was significantly delayed. Serum interleukin-6 was elevated in IL-6+/+ mice following ultraviolet irradiation, with peak concentration at 4 h, but no increase in serum interleukin-1β was found in either IL-6+/+ or IL-6–/– mice. Interestingly, tumor necrosis factor α concentration in serum was elevated at 12 h postirradiation in IL-6+/+ mice, but there was an earlier (at 4 and 8 h) time-dependent increase in tumor necrosis factor α in serum of the IL-6–/– mice. Skin zinc and copper concentrations were not altered by ultraviolet irradiation in either IL-6+/+ or IL-6–/– mice. The results suggest that interleukin-6 may be a very early mediator of cutaneous metallothionein induction by ultraviolet radiation, but that this role is possibly assumed by alternative cytokines like tumor necrosis factor α when interleukin-6 is deficient. ***Key words: cell proliferation/cytokine/PCNA/TNF. J Invest Dermatol 114:343–348, 2000***

Metallothionein (MT) is a unique heat-stable low molecular weight (6 kDa) protein without aromatic amino acids, containing 20 cysteine residues. Because of high affinity to bind to and be induced by essential metals such as zinc and copper and harmful metals like cadmium and mercury, the protein is believed to play an important role in the homeostatic function of essential metals and in detoxification against toxic metals. In previous studies MT has been revealed to localize in the skin of mouse and human (Karasawa *et al*, 1991; Van den Oord and De Ley, 1994). Anstey *et al* (1996) demonstrated ultraviolet (UV)-induced MT production in human skin, suggesting a photoprotective role of MT in the skin. MT has been suggested to be involved in defense mechanisms in the skin in response to foreign factors such as a variety of pathogens, chemicals and UV radiation. In an earlier paper, using fibroblast and keratinocyte cells from skin (Kobayashi *et al*, 1994), we reported the highest cellular MT content in the UV-resistant cell line, in contrast to the lowest MT level in the UV-sensitive cell, and induction of MT by exogenous stimulation resulted in increased tolerance against UV compared to nonstimulated cells. In mice, subcutaneous injection of cadmium, known to be a strong inducer of MT, has been reported to reduce UV toxicity effectively (Hanada *et al*, 1991).

MT produced in the acute-phase response has been proposed to act as a free radical scavenger (Karin *et al*, 1985), and we have demonstrated immunohistochemically that MT localizes in inflammatory cells infiltrating into the dermis after topical treatment by a tumor promoter (Karasawa *et al*, 1991). Since MT is quickly induced in the acute-phase response, and certain cytokines including interleukin-1β (IL-1β), tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) are reported to upregulate the synthesis of MT, much attention has been given to determining the ways in which these cytokines may interact to induce MT. IL-6, but not IL-1β, is a major cytokine for MT induction in hepatocytes (Schroeder and Cousins, 1990) and in mouse brain (Hernandez *et al*, 1997). In contrast, IL-1β, but not IL-6, is reported to be a strong MT inducer in a human astrocytoma cell line (Kikuchi *et al*, 1993). MT was found to be induced by IL-1 in cultured human cells (Karin *et al*, 1985). On the other hand, IL-1, TNF-α, and IL-6 were shown to have the capacity for MT induction in cultured vascular endothelial cells (Kaji *et al*, 1993) and in mouse liver (Liu *et al*, 1991). Tissue-specific cytokine expression for MT induction was reported by De *et al* (1990), who showed that TNF-α and IL-6 were effective MT inducers only in liver. Interactions among these cytokines make it difficult to elucidate which cytokine acts as the main mediator of MT induction.

In this study we have used IL-6–/– mice, generated by gene targeting, as a model to clarify the effect of IL-6 on MT

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Reprint requests to: Dr. Noriko Nishimura, Environmental Health Sciences Division, National Institute for Environmental Studies (NIES), 16–2 Onogawa, Tsukuba, Ibaraki 305–0053, Japan.

Abbreviations: IL-6–/– mouse, interleukin-6 null mouse; IL-6+/+ mouse, wild-type mouse; MT, metallothionein; PCNA, proliferating cell nuclear antigen.

production, and UV irradiation of the skin as an exogenous stimulant, since UV irradiation induces the release of various cytokines, including IL-6 (see review by Ullrich, 1995). The aim of this study was therefore to determine a possible involvement of IL-6 and related cytokines in cutaneous MT induction and in protection from skin damage caused by UV irradiation.

MATERIALS AND METHODS

Animals IL-6 null mice (IL-6–/–), and B6J129Sv mice (IL-6+/+) as controls, were purchased from the Jackson Laboratories (Bar Harbor, Maine, ME) and bred in the National Institute for Environmental Studies. IL-6–/– mice were viable, fertile, and phenotypically normal. Mice were maintained in standard conditions under a 12 h light-dark cycle, 23 ± 1°C, humidity 55% ± 10%. Female mice aged 8 wk were used, and hair was shaved from the entire dorsal area with a fine blade Thrive hair clipper (model 6000AD, Daito Electric Machine Industry, Osaka, Japan), leaving only faint stubble. We selected mice in the resting phase of hair growth and irradiated with ultraviolet (UV) 24 h after hair removal as described below. Hair was also clipped for unirradiated mice as well.

UV irradiation The UV irradiation was provided by unfiltered fluorescent UVB tubes (F15T8.UV-B, 280–320 nm, Sankyo, Japan). Irradiance was measured at 310 nm using a UVX radiometer (UV Products, San Grabriel, CA) and was found to be 0.4 mW per cm^2 at the target distance (35 cm). Mice received a 30 min exposure, approximately equal to three times the minimal edematous dose, a dose of 7.2 kJ per m^2, as described earlier (Nishimura *et al*, 1999).

MT and metal analysis Dorsal skin samples were collected from three mice of each group at 0 (unirradiated), 12, 24, 48, and 72 h after UVB irradiation, and were immediately frozen in liquid nitrogen. They were crushed with a high powered press (Cryo-press CP-100 W, Microtec, Chiba, Japan) and were homogenized at the time of analysis. MT was determined by radioimmunoassay as described earlier (Nishimura *et al*, 1990). Cutaneous zinc and copper concentrations were determined by inductively coupled plasma emission spectrometry (ICP; model ICAP-750, Nippon-Jarrel-Ash, Tokyo, Japan) after digesting samples with an acid mixture (HNO_3:$HClO_4$ = 3:1).

Serum IL-6, IL-1β, and TNF-α measurements Cytokine amounts in serum samples of five mice from each group were measured at 0 (unirradiated), 4, 8, and 12 h after UV exposure, using BIOTRAK ELISA kits (Amersham Pharmacia Biotech, Buckinghamshire, U.K.), according to the manufacturer's instructions. Results were expressed as pg per ml ± SEM.

Statistical analyses were performed using one-way analysis of variance followed by Fisher's least significant difference tests for *post hoc* comparison. Differences between groups were considered significant at $p < 0.05$. Comparisons of mean values between IL-6–/– and IL-6+/+ mice were performed by Student's *t* test.

Immunohistochemical staining Dorsal skin samples were collected from three mice of each group at 0 (unirradiated), 12, 24, 48, and 72 h after UV irradiation and fixed in HistoChoice (Amresco, Parkway, OH) followed by embedding with paraffin. Deparaffinized 5 μm tissue sections were subjected to immunohistochemical staining for MT. The bound anti-rat MT antibody was allowed to react with the avidin-biotin peroxidase complex immunostaining method (PK-4000, Vector Laboratories, Burlingame, CA) to visualize tissue MT as described in our earlier paper (Nishimura *et al*, 1989b). Non-immune rabbit serum was used for a negative control test. For proliferating cell nuclear antigen (PCNA) staining, deparaffinized tissue sections were treated with 3% hydrogen peroxide solution for 5 min. The slides were incubated with DAKO EPOS Anti-PCNA/HRP (DAKO, Glostrup, Denmark) for 1 h at room temperature. After rinsing with phosphate-buffered saline, the sections were incubated with diaminobenzidine for 5 min and counterstained with hematoxylin. The control section was treated with normal mouse serum instead of DAKO EPOS Anti-PCNA/HRP.

RESULTS

Metallothionein, zinc, and copper levels of IL-6–/– and IL-6+/+ mouse skin after UV irradiation As shown in **Fig 1**(*a*), almost no MT was detected in either IL-6–/– or IL-6+/+ strains before the treatment. Cutaneous MT in IL-6+/+ mice was induced by 12 h, and the time-dependent increase continued at least up to 72 h after UV exposure. UV irradiation of the IL-6–/– mice, however, did not affect cutaneous MT levels by 24 h. There was delayed induction of MT at 48 h postirradiation, at which time the cutaneous level of MT in IL-6–/– mice was the same level as that of IL-6+/+ mice.

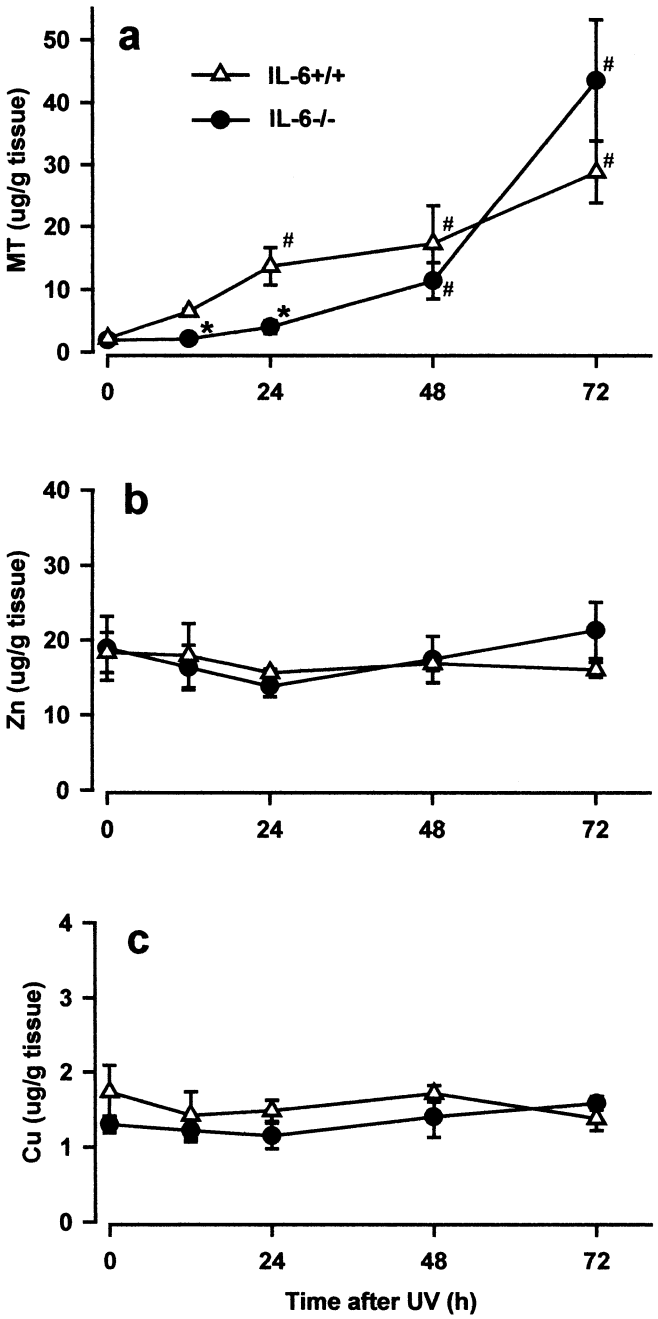

Figure 1. Delayed induction of MT in the IL-6–/– mouse skin without alteration of zinc and copper levels after UV irradiation. MT was induced by 12 h following UV exposure in the IL-6+/+ mouse skin, whereas no induction of MT was observed by 24 h post-UV irradiation in IL-6–/– mouse skin. By 48 h post-UV irradiation, MT was induced in IL-6–/– mouse skin (*a*). Cutaneous levels of zinc (*b*) and copper (*c*) were not affected by UV irradiation. Data indicate the mean ± SEM for three mice. Symbols (*, #) indicate a statistically significant difference ($p < 0.05$) from IL-6+/+ mice and the corresponding 0-time control, respectively.

Metallothionein is induced by zinc or copper, and we investigated whether or not MT induction observed in UV-treated mice skin might be a secondary effect resulting from alteration of the metal concentrations. The concentration of the two metals in

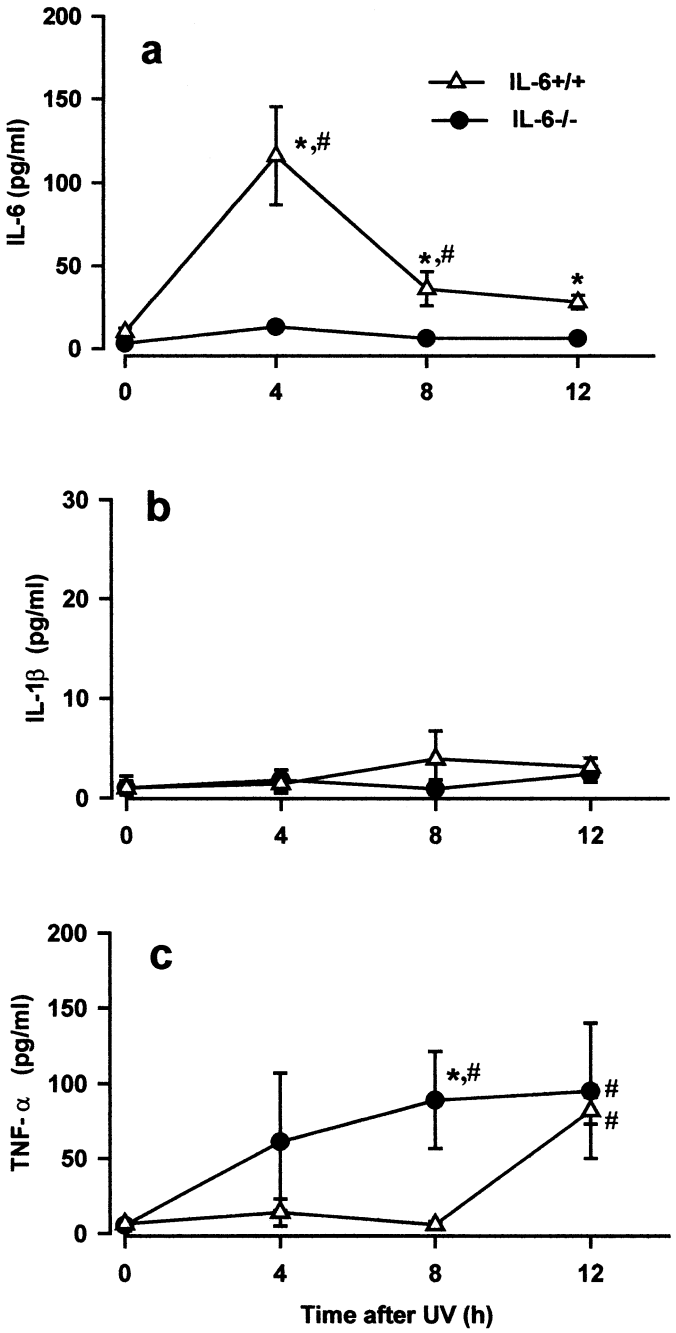

Figure 2. Rapid induction of serum IL-6 in IL-6+/+ mice, no change in IL-1, and time-dependent increase in TNF-α levels after UV irradiation. Serum IL-6 was dramatically induced as early as 4 h after UV irradiation in IL-6+/+ mice, but not detectable in IL-6–/– mice (*a*). UV irradiation did not influence serum IL-1β levels in either IL-6+/+ or IL-6–/– mice (*b*). Serum TNF-α levels were increased time-dependently in IL-6–/– mice after UV irradiation. Note the higher level in IL-6–/– than in IL-6+/+ mice (*c*). Data indicate the mean ± SEM for five mice. Symbols (*, #) indicate a statistically significant difference ($p < 0.05$) from IL-6–/– mice (*a*) or IL-6+/+ mice (*c*), and the corresponding 0-time control, respectively.

the skin of IL-6–/– and IL-6+/+ mice did not change after UV exposure (**Fig 1*b*, *c***).

Serum levels of IL-6, IL-1β, and TNF-α for IL-6–/– and IL-6+/+ mice after UV irradiation We next examined the time course of serum cytokine levels after UV irradiation. IL-6+/+ mice produced a rapid and dramatic induction of serum IL-6 by 4 h after UV irradiation, followed by a quick decrease. Serum IL-6 was not detected in IL-6–/– mice at any time before and after the irradiation (**Fig 2*a***). Unlike IL-6, UV irradiation had no effect on the IL-1β concentration in serum at any time after exposure, in either IL-6+/+ or IL-6–/– mice (**Fig 2*b***). A change in serum TNF-α content after UV irradiation is shown in **Fig 2(*c*)**. There was a time-dependent increase in serum TNF-α concentration in IL-6–/– mice. On the other hand, in IL-6+/+ mice, no appreciable induction of TNF-α level was observed by 8 h, but it reached almost the same level as in IL-6–/– mice at 12 h post-UV irradiation.

Immunohistochemical localization of MT and PCNA in the skin of IL-6–/– and IL-6+/+ mice after UV irradiation The negative controls, stained with the nonimmune rabbit serum as the first antibody, showed no specific reaction. Skin from untreated mice, whether IL-6–/– or IL-6+/+, showed no immunopositivity for MT staining (data not shown), in agreement with the radioimmunoassay (**Fig 1*a***). MT was found to appear first in fibroblasts in the dermis of IL-6+/+ mouse skin by 24 h after UV irradiation (**Fig 3*a***). The number of positive cells for MT staining was then markedly increased, particularly in inflammatory cells such as fibroblasts, neutrophils, and macrophages (**Fig 3*b***). It is worth noting that strong staining for MT in the cells of the basal layer of the hyperplastic epidermis became conspicuous at 72 h in the UV-treated IL-6+/+ mice (**Fig 3*c***). In contrast, no MT was found by 24 h (**Fig 3*d***) in IL-6–/– mouse skin, but weak MT staining was detected in the fibroblasts by 48 h after UV exposure (**Fig 3*e***), followed by an increase until 72 h. MT immunoreactivity in basal cells was detected as early as 72 h post-UV in the IL-6–/– mouse skin (**Fig 3*f***). These immunohistochemical findings were in agreement with the quantitative analysis of MT (**Fig 1*a***).

To examine whether IL-6 is necessary for stimulating the proliferative response in hyperplasia induced by UV irradiation, immunostaining for PCNA was performed. The negative controls were stained with the nonimmune mouse serum as the first antibody, and showed no specific reaction. Skin from untreated mice, whether IL-6–/– or IL-6+/+, showed almost no immunopositive staining (data not shown). PCNA-labeled keratinocytes were found in the basal cell layer at 24 h after UV irradiation in the epidermis of IL-6+/+ mice (**Fig 4*a***). With increase in hyperplasia, the cell density and intensity of PCNA staining became stronger (**Fig 4*b***) and the activity reached its maximum at 72 h (**Fig 4*c***). On the other hand, almost no activity was found in the UV-irradiated IL-6–/– mouse skin by 24 h (**Fig 4*d***). The staining intensity of PCNA gradually increased in the epidermis by 48 h, but the skin thickness was noted to be less than that of the IL-6+/+ mice (**Fig 4*e***). An equivalent staining intensity to IL-6+/+ mouse skin was found by 72 h after UV exposure in IL-6–/– mouse skin (**Fig 4*f***). PCNA immunoreactivity was found to localize predominantly in basal and suprabasal cells whereas MT staining with cytoplasmic staining was restricted to the basal layer of the epidermis (**Figs 3*c*, 4*c***).

DISCUSSION

In this study of the role of IL-6 in MT induction stimulated by UV exposure of the skin, we have shown that cutaneous MT level quantitated by radioimmunoassay correlates very well with the intensity of MT immunostaining in the skin tissue. We have demonstrated a rapid MT induction by 12 h in the skin of IL-6+/+ mice, but a delayed MT induction in IL-6–/– mouse skin until after 24 h. The delayed MT induction in IL-6–/– mouse skin suggests that IL-6 could be a critical early cytokine to initiate MT synthesis, but that in IL-6–/– mice its role may be assumed by other cytokine(s) known also to be induced by UV radiation, such as IL-1β and TNF-α. As shown in **Fig 2(*b*)**, there was no difference in the IL-1β level in the serum of either the IL-6–/– or IL-6+/+ mice following UVB exposure. On the other hand, TNF-α showed a time-dependent increase in the serum of IL-6–/– mice immediately

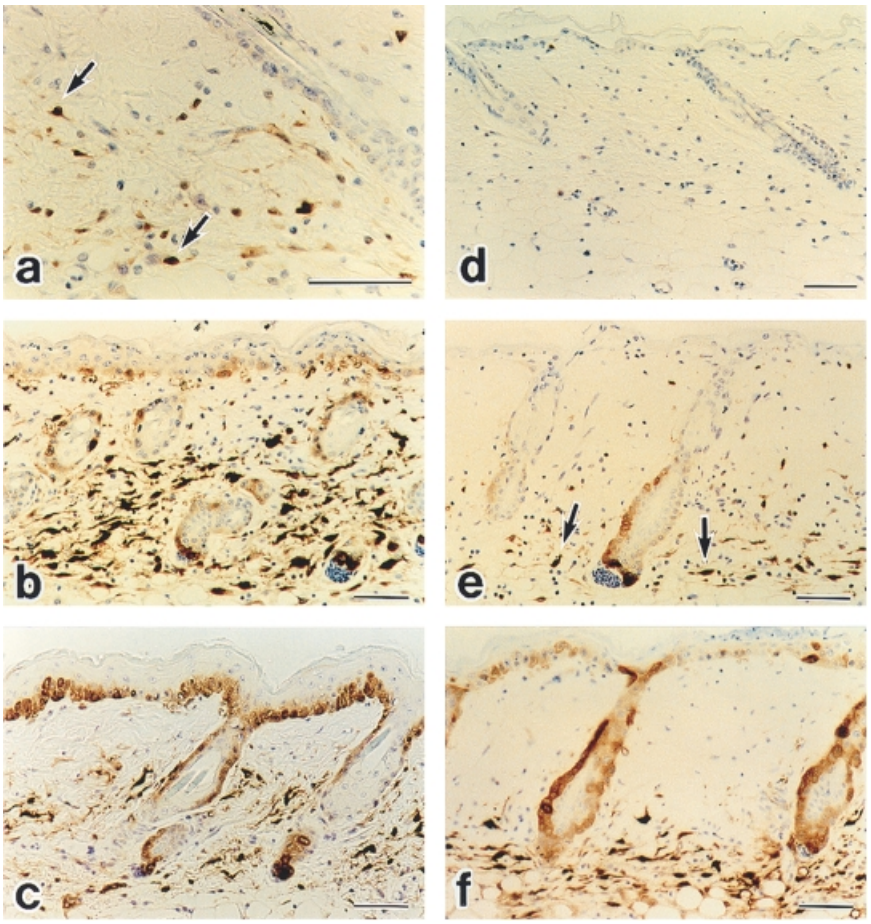

Figure 3. MT induction in fibroblasts and keratinocytes in IL-6+/+ and IL-6–/– mice after UV. Upon UV irradiation, MT positive fibroblasts (arrows) appeared by 24 h (*a*) and MT was detectable in basal cells of hyperplastic epidermis by 48 h (*b*) and 72 h (*c*) in IL-6+/+ mouse skin. On the other hand, in IL-6–/– mouse skin, no MT staining was found by 24 h (*d*), and MT localization in fibroblasts (arrows) appeared by 48 h after UV exposure (*e*). MT was detectable in basal cells at 72 h (*f*) after UV irradiation. Less hyperplastic epidermis was evident in IL-6–/– mouse skin compared with IL-6+/+ mice at any time examined after UV exposure. *Scale bar*: 50 μm.

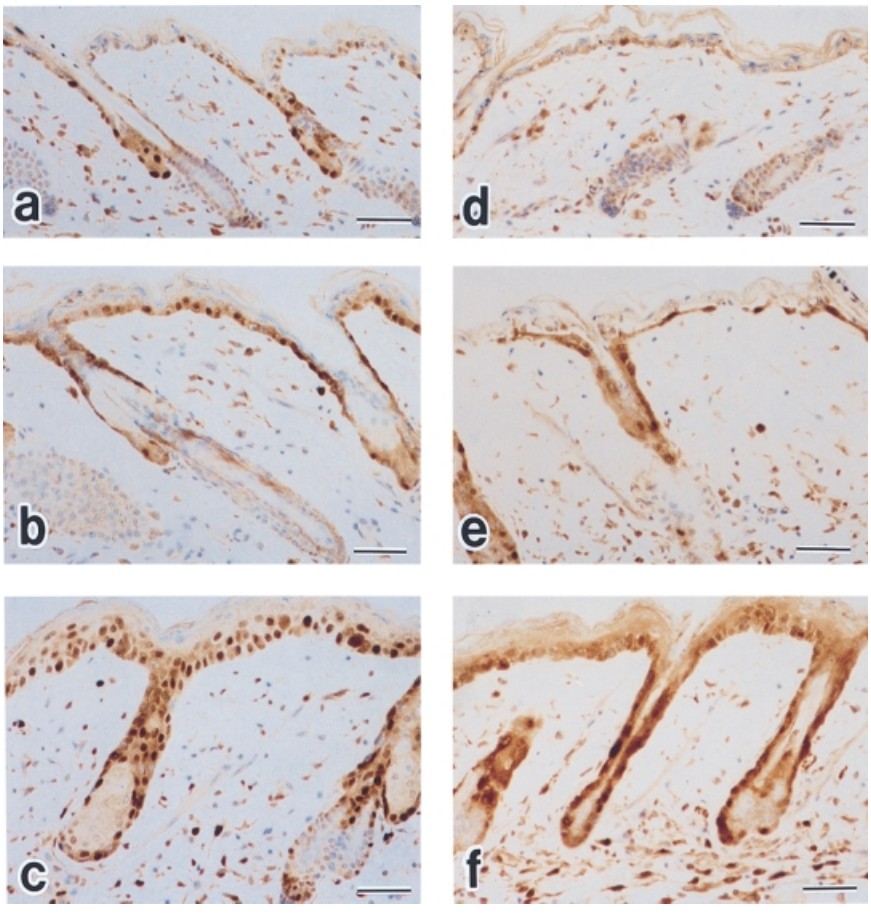

Figure 4. Active cell division in IL-6+/+ mouse skin induced by UV irradiation. PCNA positive basal cells were clearly seen by 24 h (*a*) and the intensity of the staining became greater at 48 h (*b*) and reached a maximum at 72 h (*c*) following UV exposure in IL-6+/+ mouse skin. No immunostaining for PCNA was found by 24 h (*d*) in IL-6–/– mouse skin. PCNA-labeled basal cells were detectable by 48 h (*e*) and 72 h (*f*) following UV exposure. *Scale bar*: 50 μm.

after UV exposure, but an increase in TNF-α was apparent only later at 12 h in IL-6+/+ mice. This observation suggests that, when IL-6 was deficient, an earlier appearance of TNF-α could compensate for the activation of MT induction in the skin. Inter-regulatory actions of these cytokines associated with MT induction have been reported previously for the liver (De *et al*, 1990; Liu *et al*, 1991) and vascular endothelium (Kaji *et al*, 1993), and a similar compensation mechanism for MT induction was found in the remaining liver from partially hepatectomized IL-6 null mice (Molotkov *et al*, 1999). The results presented here indicate that, for the skin, IL-6 acts as an early cytokine for MT synthesis, but can be compensated for by TNF-α, and that in spite of reports of IL-1 activity for MT induction in other tissues (Karin *et al*, 1985; De *et al*, 1990; Liu *et al*, 1991; Sato *et al*, 1992; Huber and Cousins, 1993; Kaji *et al*, 1993; Kikuchi *et al*, 1993; Hernandez and Hidalgo, 1998), IL-1 does not appear to be important in this role in the skin. In coming to this conclusion, we assume that the cytokines appearing in the serum were originated from the UV-irradiated skin, where their actions on MT would be direct. In support, we have previously observed UV-induced IL-6 in both skin and serum of the mouse (Nishimura *et al*, 1999), and other reports also correlate skin cytokine activity with serum levels (Ullrich, 1995). This assumption should be confirmed in future studies, however.

Four MT isoforms have been described, having differential expression in various organs and different responses to stimuli (Andrews, 1990; Uchida *et al*, 1991; Quaife *et al*, 1994). It is interesting that the most recently discovered MT-IV was found by *in situ* hybridization to localize in epithelial tissue of the tongue, and in neonatal mouse skin, but MT-IV has not been described in adult skin, nor has cross-reactivity with antibody to MT-I and MT-II been measured. In the tongue, MT-IV mRNA was localized in the differentiating spinous layer (Quaife *et al*, 1994), whereas we found MT-I and MT-II protein restricted to the basal layer of mouse skin, thus making it unlikely that, if MT-IV were present, the antibody to MT-I and MT-II would detect it.

Zinc and copper effectively induce MT (see reviews by Hamer, 1986; Andrews, 1990), and MT gene expression in various tissues has been suggested to respond to zinc and copper status (Blalock *et al*, 1988; Huber and Cousins, 1993). We thus examined the possibility of a secondary effect on MT induction resulting from altered endogenous levels of zinc and copper. The present study showed that the levels of neither metal were altered by UV exposure, suggesting no involvement of zinc or copper in enhancement of cutaneous MT levels. This observation that MT induction by UV irradiation is not mediated by metals is consistent with a study with X-irradiated animals (Shiraishi *et al*, 1986; Koropatnick *et al*, 1989).

Unlike with IL-6+/+ mice, UV irradiation of IL-6–/– mice did not cause epidermal hyperplasia by 48 h, and only a slight degree of hyperplasia at 72 h. As shown in **Fig 4**, the PCNA staining of the UV-irradiated IL-6+/+ keratinocytes was progressively greater in intensity and cell density than for IL-6–/– mouse skin. This suggests direct involvement of IL-6 in the proliferation of keratinocytes, which is in agreement with the observation of actively expressed IL-6 gene in the hyperplastic proliferative lesions of psoriatic skin (Grossman *et al*, 1989). Addition of rIL-6 to cultures of normal human keratinocytes also resulted in a 7-fold increase in tritiated thymidine incorporation. A possible role of MT in cell division possibly by supplying zinc to zinc-requiring enzyme has been suggested (Nishimura *et al*, 1989a; Karasawa *et al*, 1991; Tohyama *et al*, 1993; Hanada *et al*, 1998). From our data for the mouse skin, we postulate that IL-6 released from inflammatory cells or fibroblasts following UV exposure may act as a causative factor for the cutaneous biosynthesis of MT, whose function is to supply zinc to enzymes involved in keratinocyte proliferation.

In conclusion, we have demonstrated that UVB-stimulated MT induction in mouse skin was delayed in the absence of IL-6. The dependence on IL-6 did not appear to be associated with IL-1 levels, but could be compensated at least partly by TNF-α. Based on our histological findings, we suggest that IL-6 is an essential cytokine to initiate the keratinocyte proliferation associated with the hyperplastic response to UV irradiation, in which the synthesis of MT in the skin plays an important role.

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