A role for NF-κB–dependent gene transactivation in sunburn

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Exposure of skin to ultraviolet (UV) radiation is known to induce NF-κB activation, but the functional role for this pathway in UV-induced cutaneous inflammation remains uncertain. In this study, we examined whether experimentally induced sunburn reactions in mice could be prevented by blocking UV-induced, NF-κB–dependent gene transactivation with oligodeoxynucleotides (ODNs) containing the NF-κB *cis* element (NF-κB decoy ODNs). UV-induced secretion of IL-1, IL-6, TNF-α, and VEGF by skin-derived cell lines was inhibited by the decoy ODNs, but not by the scrambled control ODNs. Systemic or local injection of NF-κB decoy ODNs also inhibited cutaneous swelling responses to UV irradiation. Moreover, local UV-induced inflammatory changes (swelling, leukocyte infiltration, epidermal hyperplasia, and accumulation of proinflammatory cytokines) were all inhibited specifically by topically applied decoy ODNs. Importantly, these ODNs had no effect on alternative types of cutaneous inflammation caused by irritant or allergic chemicals. These results indicate that sunburn reactions culminate from inflammatory events that are triggered by UV-activated transcription of NF-κB target genes, rather than from nonspecific changes associated with tissue damage.

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Introduction

Ultraviolet (UV) radiation causes sunburn reactions, immunosuppression, accelerated skin aging, and skin cancer, and it is considered to be an important environmental hazard for humans (1). At cellular levels, UV radiation has been shown to trigger cytokine production (2), regulate surface expression of adhesion molecules (3), affect cellular mitosis (4), and induce apoptotic cell death (5, 6). With respect to molecular mechanisms, UV radiation is known to alter cellular function via DNA damage (7–10), generation of reactive oxygen intermediates (ROIs) (11, 12), ligand-independent signaling through cell surface receptors, such as EGF, TNF, and IL-1 receptors (13–15), phosphorylation of receptor-associated tyrosin kinases and protein kinase C (15–18), and activation of selected transcription factors, including NF-κB, AP-1, and AP-2 (13, 19–21). These mechanisms are by no means mutually exclusive; rather, they are likely to operate in an interdependent manner. For example, NF-κB activation can be induced experimentally by damaging DNA chemically (6), exposure to hydrogen peroxide (22), clustering of surface receptors (13), or phosphorylation of tyrosin kinases or protein kinase C (17, 23, 24). NF-κB activation, in turn, leads to the expression of many genes involved in immunological and inflammatory responses, including genes that encode cytokines, adhesion molecules, and regulators of cell growth and death (24, 25). Thus, we sought to determine the causative relationship between UV-dependent NF-κB activation and the development of sunburn reactions.

For this aim, we used the recently developed technology of decoy ODNs. The original concept of using synthetic double-stranded ODN as "decoy" *cis* elements to block the binding of nuclear factors to promoter regions of target genes was introduced in 1990 by Sullenger et al. and Bielinska et al. (26, 27). In 1997, Morishita et al. reported the first in vivo application of this technology; they prevented myocardial infarction after reperfusion in rats by direct infusion of synthetic double-stranded 20-bp ODNs containing the NF-κB *cis* element into cannulated coronary arteries (28). Subsequently, Ono et al. prevented cerebral angiopathy after subarachnoid hemorrhage in rabbits by delivering NF-κB decoy ODNs into the subarachnoid space (29), and Kawamura et al. showed that direct injection of NF-κB decoy ODNs into implanted tumors in mice inhibited cachexia, without affecting the tumor growth (30). Here we report the first application to our knowledge of the decoy ODN technology to the skin (as a target organ) and to prevent inflammatory responses to an environmental hazard (as a target disease).

Methods

Animals and cell lines. Female BALB/c mice (4–6 weeks old) were housed in the pathogen-free facility of the Animal Resource Center at the University of Texas Southwestern Medical Center, and all the animal experiments were approved by the Institutional Review Board and conducted according to guidelines of the National Institutes of Health (NIH). The Pam 212 keratinocyte line (established from BALB/c mouse skin) (31) and the NS47 fibroblast line (from BALB/c mouse skin) (32) were maintained and expanded in complete RPMI 1640 containing 10% FCS (32). The XS106 Langerhans cell line (from A/J mouse skin) (33) was cultured in complete RPMI 1640 in the presence of 1 ng/mL mouse recombinant GM-CSF and 10% (vol/vol) NS47 fibroblast culture supernatant as described previously (34).

UV radiation. The dorsal surfaces of unrestrained BALB/c mice were exposed from above to a bank of four unfiltered FS20 sunlamps (Westinghouse, Pittsburgh, Pennsylvania, USA) that emit a spectrum with high irradiance in the UVB region and a peak at 313 nm (35). Cell lines cultured in 100-mm tissue culture plates were washed twice with PBS and then exposed to either FS20 sunlamps or a solar simulator with 1 kW Xenon arc lamp (Model 81190; Oriel Instruments, Stratford, Connecticut, USA). An IL 700 research radiometer equipped with a SEE 240 photodetector (International Light Inc., Newburyport, Massachusetts, USA) was used to measure the irradiance in the UVB region (sunburn spectrum) for both light sources.

NF-κB decoy ODN treatment. Phosphorothioated, double-stranded 20-bp NF-κB decoy ODNs (5′-CCTTGAAGGGATTTCCCTCC-3′) and scrambled ODNs (5′-TTGCCGTACCTGACTTAGCC-3′) were synthesized according to Morishita et al. (28). NF-κB decoy ODNs or scrambled ODNs (0.5 mM solution in PBS) were injected intraperitoneally (200 μL/injection per mouse) 24 hours and 1 hour before irradiation. In some experiments, mice received a single intraperitoneal injection (200 μL/mouse) 6 hours before irradiation. For local administration, ODN preparations (10 μM) were injected subcutaneously (20 μL/injection per animal) into the dorsum of mouse ear skin 6 hours before irradiation. For topical application, mouse ears were first wiped gently six times with acetone to remove the barrier effect of stratum corneum. Subsequently, 10 μM ODN preparations were applied topically (20 μL/ear) onto the dorsal aspects of treated ears. In some experiments, NF-κB decoy ODNs were applied onto ear skin to test their impact on irritant contact hypersensitivity reactions to croton oil (36) and allergic contact hypersensitivity reactions to oxazolone (34). For in vitro experiments, cells were preincubated for 6 hours with 10 μM NF-κB decoy ODNs or scrambled ODNs, washed twice with PBS, exposed to UV radiation, and then cultured in complete RPMI (without GM-CSF or NS supernatants) in the presence of freshly added ODN for an additional 24 hours. To study the pharmacokinetics, XS106 cells were incubated with 10 μM FITC-conjugated NF-κB decoy ODN or FITC-conjugated scrambled ODN at either 4°C or 37°C and then analyzed for the uptake of FITC-conjugated ODN probes by FACS or under a Leica CLSM laser scanning confocal microscope (Leica, Deerfield, Illinois, USA) as described previously (37).

Assays to assess the impact of NF-κB decoy ODNs. Before and after UV irradiation, ear skin thickness was measured by a third experimenter "blinded" to sample identity, using a caliper-type engineer's micrometer (34). Some ear skin samples were fixed in formalin for histological evaluation. Thickness of ear skin sections, number of keratinocyte layers in the epidermis, number of infiltrating leukocytes, and frequency of sunburn cells were measured by light microscopy after hematoxylin and eosin (H&E) staining. To determine the impact of NF-κB decoy ODN on UV-induced DNA damage, ear skin samples were harvested 5 minutes after UV radiation, and ear skin sections were examined for cyclobutane pyrimidine dimers (CPDs) by using mAb H3 (38) and FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA) as described previously (39). Surface densities of Langerhans cells in ear skin were examined 24 hours after UV radiation as described elsewhere (40). Briefly, the epidermis was separated as an intact sheet from the remaining dermis after a 20-minute incubation in 0.5 M ammonium thiocyanate at 37°C, fixed in acetone, and then stained with FITC-conjugated anti-IA mAb 2G9 (PharMingen, San Diego, California, USA). Numbers of IA-positive epidermal cells (i.e., Langerhans cells) were counted under a fluorescence microscope. Some ear specimens were extracted in 10 mM HEPES/10 mM KCl buffer (pH 7.9) with 0.08% NP-40, 0.1 mM EDTA, 1 mM EGTA, 0.5 mM DTT, and 0.5 mM PMSF, and the resulting soluble fractions were then examined for cytokines by using ELISA kits (R&D Systems Inc., Minneapolis, Minnesota, USA). To study in vitro efficacy, all cultures were harvested 24 hours after UV irradiation. Cells were examined for viability by trypan blue exclusion and by a FACS-based propidium iodine assay (35), whereas culture supernatants were tested for cytokines by ELISA. In some experiments, cells were transfected with the NF-κB reporter construct (3× κB luc) or the AP-1 reporter construct (4× AP-1 luc) (41, 42), both of which were kindly provided by A. Weiss (University of California, San Francisco, California, USA). After 6 hours of preincubation with 10 μM NF-κB decoy or scrambled ODNs, cells were washed and then exposed to UV radiation. Subsequently, the cells were cultured for an additional 24 hours in the continuous presence of freshly added 10 μM NF-κB decoy or scrambled ODNs and examined for luciferase (Luc) activities as before (33).

Statistical analyses. Animal experiments were performed with five to ten mice per group, and in vitro experiments were performed with triplicate samples.

All experiments were repeated at least three times to assess reproducibility, and the data were evaluated for statistical significance by the Student's *t* test.

Results

In vitro effect of NF-κB decoy ODN on UV-induced secretion of proinflammatory cytokines by skin-derived cell lines. In the first set of experiments, we irradiated three skin-derived cell lines with conventional FS20 sunlamps and examined NF-κB activation by the gel shift assays. A single exposure to a sublethal dose (50 J/m^2) resulted in significant NF-κB activation in all cell lines (Pam 212 keratinocyte, NS47 fibroblast, and XS106 Langerhans cell lines), and in vitro binding of radiolabeled NF-κB probe to the nuclear extracts isolated from UV-irradiated cells was competed with "cold" NF-κB probe, as well as with phosphorothioated, double-stranded 20-bp NF-κB decoy ODN (data not shown). To determine the function of the NF-κB decoy ODN at cellular levels, we next transfected each cell line with the NF-κB reporter construct (3× κB luc), in which the Luc gene was inserted downstream from the NF-κB–binding motifs. As shown in Figure 1a, UV radiation at 50 J/m^2 (FS20 sunlamps) elevated Luc activity significantly in each cell line. Likewise, each cell line transfected with the AP-1 reporter construct (4× AP-1 luc) also responded to UV radiation by elevating Luc activity. Importantly, addition of the NF-κB decoy ODN blocked UV-induced Luc elevation with the NF-κB reporter construct almost completely, whereas scrambled ODN had no significant effect. Moreover, NF-κB decoy ODN showed minimal, if any, effects on UV-induced Luc elevation with the AP-1 reporter construct, confirming the specificity. Thus, UV radiation triggers activation of the NF-κB and AP-1 pathways in each of three skin-derived cell lines, and NF-κB decoy ODNs selectively block the NF-κB–dependent gene transactivation pathway alone.

A single exposure of the Pam 212 keratinocyte line to UV radiation at sublethal doses (50 J/m^2 with FS20 sunlamps or 100 J/m^2 with a Xenon arc solar simulator) induced the secretion of VEGF in a dose-dependent manner (data not shown). Likewise, the NS47 fibroblast line secreted primarily IL-6 and VEGF in response to radiation. The XS106 Langerhans cell line secreted TNF-α, IL-1β, and IL-6 in response to UV irradiation. Thus, three cell lines derived from skin all responded to radiation by secreting proinflammatory cytokines (albeit in different profiles), thus, providing an in vitro experimental system to study the impact of NF-κB decoy ODN on UV-induced changes in cellular function. As noted in Figure 1b, NF-κB decoy ODNs (10 μM) prevented the secretion of VEGF by Pam 212 keratinocytes, IL-6 and VEGF by NS47 fibroblasts, and TNF-α, IL-1β, and IL-6 by XS106 Langerhans cells after exposure to FS20 sunlamps (50 J/m^2). Similar inhibition was also observed for cytokine production triggered by exposure to the solar simulator (100 J/m^2) (Figure 1c). By contrast, scrambled control ODNs showed no inhibitory effect on any tested cytokine.

Consistent with the functional data described earlier here, the FITC-conjugated NF-κB decoy ODNs were incorporated into the XS106 cells in a time- and temperature-dependent fashion (Figure 2a). Importantly, the FITC-conjugated scrambled ODNs were also incorporated with similar kinetics and efficiency, thus, vali-

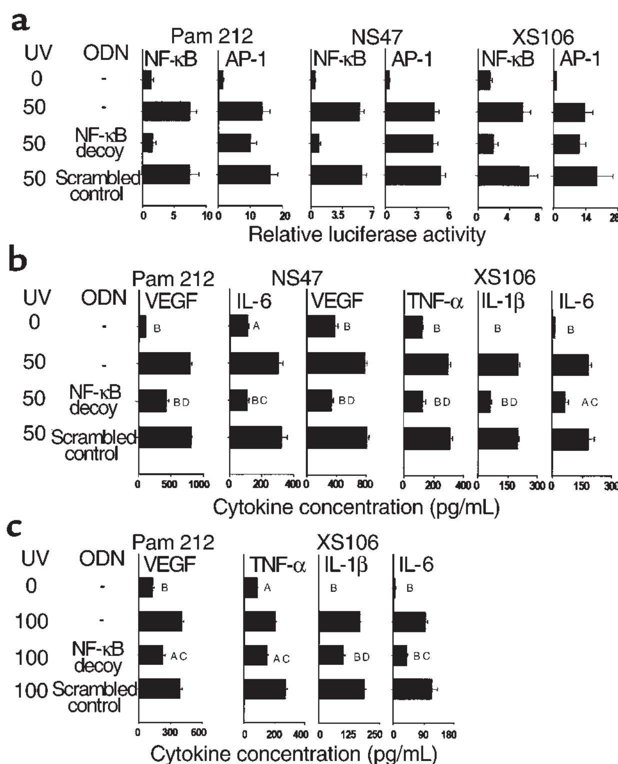

Figure 1
Impact of NF-κB decoy ODNs on UV-triggered cytokine production. (**a**) Pam 212 keratinocytes, NS47 fibroblasts, and XS106 Langerhans cells were transfected with the NF-κB reporter construct (3× κB luc) or the AP-1 reporter construct (4× AP-1 luc). After 6 hours of preincubation with 10 μM NF-κB decoy or scrambled ODNs, cells were washed and exposed to 50 J/m^2 UVB radiation (FS20 sunlamp) in PBS, cultured for an additional 24 hours in the continuous presence of fresh ODNs, and then examined for Luc activity. Data shown are representative of four independent experiments, showing the mean ± SD from triplicate samples. (**b** and **c**) Cells (1×10^6 cells/mL) were incubated for 6 hours with 10 μM NF-κB decoy or scrambled ODNs, washed, and then exposed to the indicated fluences of UV radiation using either FS20 sunlamps (**b**) or a Xenon arc solar simulator (**c**). Subsequently, cells were cultured for an additional 24 hours in complete RPMI 1640 in the presence of fresh ODNs, and culture supernatants were examined for the indicated cytokines by ELISA. Data shown are representative of three independent experiments, showing the mean ± SD from triplicate samples. [A]Statistically significant differences ($P < 0.05$) compared with the UV-irradiated group without added ODNs. [B]Statistically significant differences ($P < 0.01$) compared with the UV-irradiated group without added ODNs. [C]Statistically significant differences compared with the UV plus scrambled ODN group ($P < 0.05$). [D]Statistically significant differences compared with the UV plus scrambled ODN group ($P < 0.01$).

dating the use of the scrambled ODNs as a control for the NF-κB decoy ODNs. Confocal microscopic analysis revealed FITC-conjugated NF-κB decoy ODN within the cytoplasm of XS106 cells after 4 hours of incubation at 37°C (Figure 2b). NF-κB decoy ODN (≤ 30 μM) showed no significant cytotoxicity for XS106 cells even when combined with sublethal irradiation (Figure 2c). Likewise, NF-κB decoy ODN had no cytotoxic effect on UV-irradiated Pam 212 cells or NS47 cells (data not shown). In dose-dependency experiments, NF-κB decoy ODNs exhibited optimal inhibition of UV-induced cytokine production at 10-30 μM (Figure 2d). Taken together, these in vitro observations indicate that UV-induced NF-κB activation is critical to UV-dependent secretion of proinflammatory cytokines, with the implication that NF-κB decoy ODN may provide a useful tool to study pathogenic mechanisms of sunburn reactions in skin.

In vivo impact of NF-κB decoy ODNs on UV-induced skin inflammation. In the preliminary experiments, we observed that a single dorsal exposure of BALB/c mouse skin to UV radiation (FS20 sunlamps) caused marked cutaneous inflammation, most remarkably on their ears, and that the extent of inflammation could be examined by measuring the ear swelling responses. We also found that a relatively large dose (3,000 J/m^2) of irradiation was required to induce experimental sunburn reactions in a reproducible and quantitative fashion. Under these conditions, ear swelling became detectable within 24 hours after irradiation, and these swelling responses progressed over the next 2–3 days (Figure 3a). Mice receiving two intraperitoneal injections of NF-κB decoy ODN 24 hours and 1 hour before UV exposure showed significantly reduced ear swelling responses at each time point tested, compared with control mice that received PBS alone or scrambled ODNs (Figure 3a). Having observed the efficacy of NF-κB decoy ODNs to prevent UV-induced skin inflammation, we next determined whether other UV-induced changes would be also affected by systemic administration of NF-κB decoy ODNs. Consistent with a previous report (43), UV radiation reduced the surface densities of epidermal Langerhans cells significantly with 60–70% reduction observed at 24 hours (Figure 3b). Two intraperitoneal injections of NF-κB decoy ODNs failed to prevent UV-induced reduction in Langerhans cell numbers compared with PBS or scrambled ODN controls, suggesting that the NF-κB pathway was not directly involved in UV-dependent Langerhans cell death and/or migration (Figure 3b). As shown in Figure 3c, UV radiation caused rapid and marked DNA damage mainly in the epidermis, as revealed by immunofluorescence staining with anti-CPD mAb. Injected NF-κB decoy ODNs had minimal effect on UV-induced CPD formation, indicating that DNA damage was not a direct consequence of UV-dependent NF-κB activation. At the same time, this implied further that DNA damage played little, if any, causative role in the development of UV-induced skin inflammation.

UV-induced ear swelling was also inhibited significantly by a single intraperitoneal injection (data not shown) or a single subcutaneous injection of NF-κB decoy ODNs into ear skin 6 hours before irradiation (Figure 4a). When mouse ear skin was first treated with acetone (to remove the barrier effect of surface stratum corneum) and then painted with FITC-conjugated NF-κB decoy ODNs, a fluorescence signal became detectable histologically in the epidermis and underlying reticular dermis in ear skin sections (data not shown). This protocol enabled us to deliver NF-κB decoy ODNs directly into the target site of UV irradiation. Topical application of NF-κB decoy ODNs was found to be at least as effective as either the systemic or local injection protocol in reducing ear swelling responses. Importantly, topically applied NF-κB decoy ODNs had no inhibitory effect on ear swelling induced by skin painting with a skin irritant chemical, croton

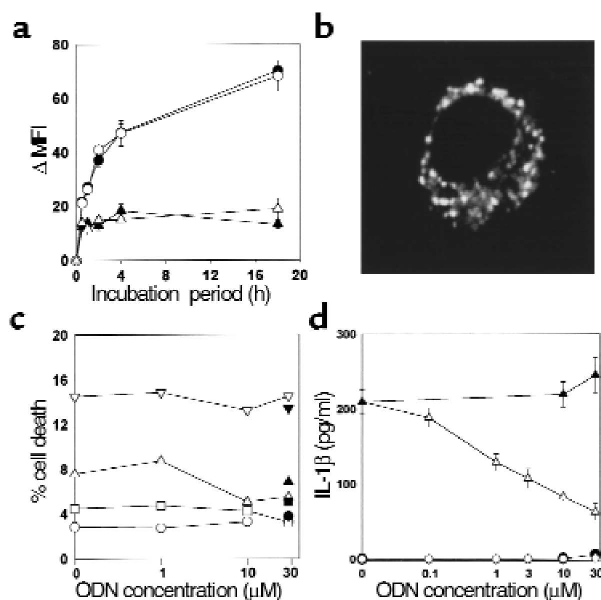

Figure 2
Pharmacokinetics of NF-κB decoy ODNs. (**a**) XS106 cells were incubated for the indicated periods with 10 μM FITC-conjugated NF-κB decoy ODNs (open symbols) or scrambled ODNs (closed symbols) at 4°C (triangles) or 37°C (circles) and then analyzed by FACS. Data shown are the mean ± SD (n = 3) of the mean fluorescence intensities (MFI). (**b**) After 4 hours of incubation with FITC-conjugated NF-κB decoy ODNs at 37°C, XS106 cells were examined by confocal microscopy. (**c**) XS106 cells were exposed to FS20 sunlamps at 100 J/m^2 (reversed triangles), 50 J/m^2 (triangles), 25 J/m^2 (squares), or 0 J/m^2 (circles), cultured in the presence of the indicated concentrations of NF-κB decoy ODNs (open symbols) or scrambled ODNs (closed symbols), and then tested for cell viability by FACS. Data shown are representative of two independent experiments. (**d**) XS106 cells were exposed to FS20 sunlamps at 50 J/m^2 (triangles) or sham-irradiated (circles), cultured for 24 hours in the presence of the indicated concentrations of NF-κB decoy ODNs (open symbols) or scrambled ODNs (closed symbols), and then examined for IL-1β secretion. Data shown are the mean ± SD from triplicate samples.

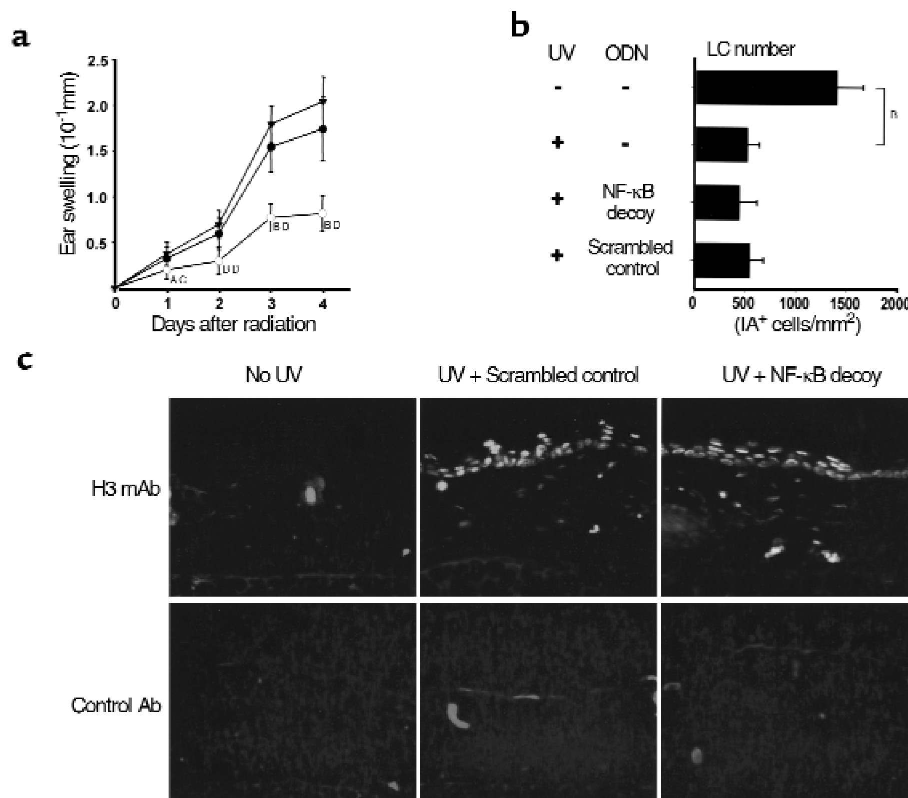

Figure 3
Inhibition of UV-induced ear skin swelling by systemic application of NF-κB decoy ODN. BALB/c mice received two intraperitoneal injections of NF-κB decoy ODN (open circles), scrambled ODNs (closed triangles), or PBS alone (closed circles) at 24 hours and 1 hour before irradiation. These animals were exposed to UV radiation and then examined for ear swelling responses at the indicated time points (**a**), surface density of epidermal Langerhans cells at 24 hours after irradiation (**b**), and CPD formation at 5 minutes after irradiation (**c**). Data shown in **a** are representative of three independent experiments, showing the mean ± SD (n = 10) of ear swelling responses (compared with ear thickness before irradiation). [A]Statistically significant differences ($P < 0.05$) compared with the UV plus PBS group. [B]Statistically significant differences ($P < 0.01$) compared with the UV plus PBS group. [C]Statistically significant differences compared with the UV plus scrambled ODN group ($P < 0.05$). [D]Statistically significant differences compared with the UV plus scrambled ODN group ($P < 0.01$). Data shown in **b** are the mean ± SD (n = 10) of numbers of IA^+ epidermal cells/mm^2 in ear skin samples. Immunofluorescence pictures shown in **c** are representative staining profiles with anti-CPD mAb H3 or with an isotype-matched control IgG. ×400.

oil, or by skin challenge with the contact sensitizer oxazolone in previously sensitized animals (Figure 4a). These observations excluded the possibility that NF-κB decoy ODN had acted simply as a nonspecific anti-inflammatory agent. In the next set of experiments, mice were exposed to UV radiation 1 hour after topical application of NF-κB decoy ODNs to their right ears. As shown in Figure 4b, ear swelling was diminished significantly in the right ears compared with PBS-treated left ears in the same UV-exposed animals, indicating that topically applied NF-κB decoy ODNs had exerted pharmacologic activity locally. In summary, skin-swelling responses to UV radiation, but not to irritant or allergic compounds, were inhibited significantly (50–70%) by systemic, local, or even topical application of NF-κB decoy ODN.

Histologically, UV-induced edema and leukocyte infiltration were both inhibited by topical application of NF-κB decoy ODN (Figure 4c). In fact, quantitative analyses of ear skin sections revealed significant reductions in ear swelling, numbers of skin infiltrating leukocytes, and extent of epidermal hyperplasia (Figure 4d). By contrast, the scrambled ODN had no inhibitory effects on any of these inflammatory parameters. Interestingly, NF-κB decoy ODN had no significant effects on the formation of sunburn cells in UV-exposed skin (Figure 4d). Thus, some, but not all, histological changes induced by UV radiation were preventable by topical application of NF-κB decoy ODNs.

To determine the impact of NF-κB decoy ODN on UV-induced accumulation of proinflammatory cytokines in vivo, we measured cytokines in whole ear skin extracts by ELISA. As observed for skin-derived cell lines in vitro (Figure 1), VEGF, TNF-α, IL-1β, and IL-6 were all elevated significantly in skin within 24 hours after UV irradiation (Figure 5a), and they remained elevated even 4 days after irradiation, when maximal ear swelling occurred (Figure 5b). Importantly, intraperitoneal injection of NF-κB decoy ODNs diminished almost completely UV-induced accumulation of these cytokines at both time points (Figure 5, a and b). Similar inhibition was also achieved by topical application

of NF-κB decoy ODNs (Figure 5c). Again, scrambled ODNs had no significant effect. Moreover, NF-κB decoy ODNs failed to affect the accumulation of the same cytokines in irritant contact dermatitis, documenting the specificity of their action (Figure 5d). These results indicate that UV-induced NF-κB activation is the critical event required for local accumulation of proinflammatory cytokines in irradiated skin.

Discussion

The present study demonstrates that UV-induced activation of NF-κB–dependent gene transactivation pathways is a critical event for the subsequent development of sunburn reactions in skin. This conclusion is supported by several lines of experimental evidence. First, UV radiation with conventional FS20 sunlamps or with a solar simulator–induced NF-κB activation in cells of

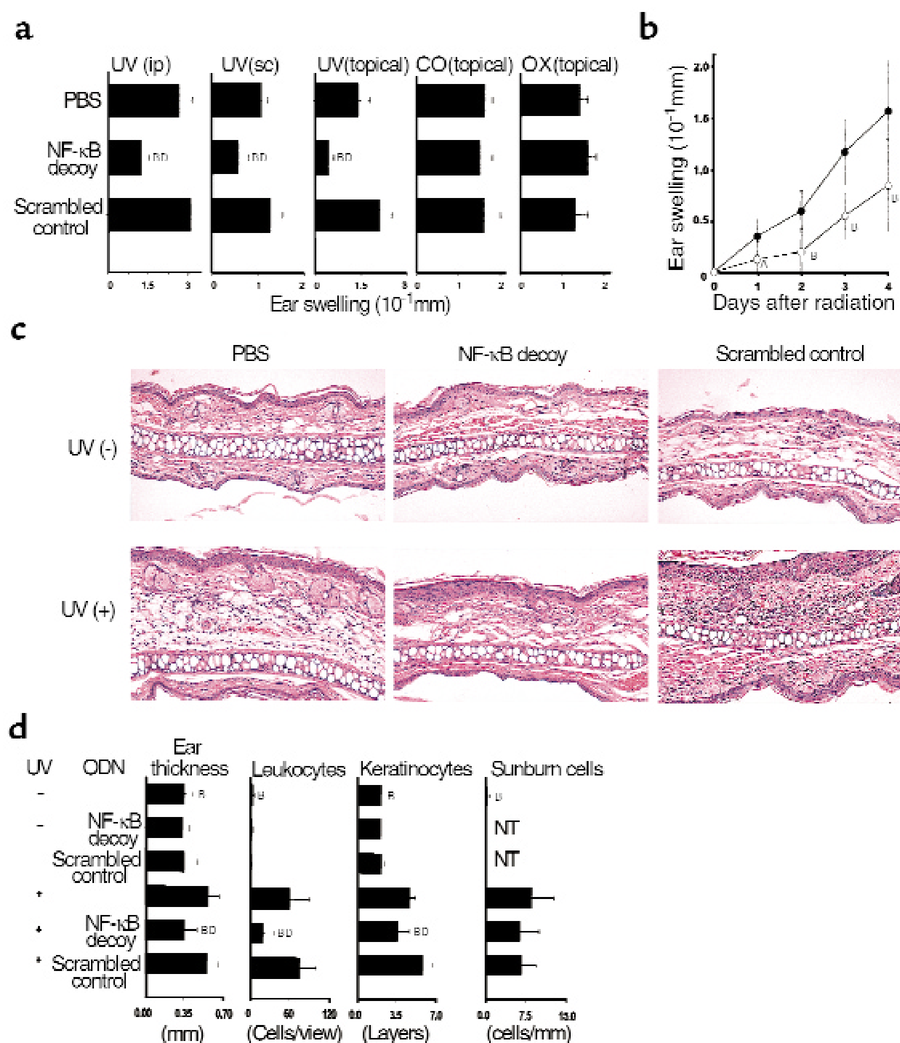

Figure 4

Impact of topically applied NF-κB decoy ODN on UV-induced inflammation. (**a**) BALB/c mice received two intraperitoneal (ip) injections, a single subcutaneous (sc) injection, or topical application of NF-κB decoy ODNs, scrambled ODNs, or PBS alone. Three groups of animals were exposed to UV irradiation, whereas two additional groups received topical application of croton oil on the ear skin or skin challenge with oxazolone (7 days after sensitization to the same hapten). Data shown are the mean ± SD (n = 10) of ear swelling responses (compared with ear thickness before treatment) at 4 days after irradiation or skin painting with croton oil (CO) or oxazolone (OX). (**b**) BALB/c mice received topical application of NF-κB decoy ODN (on right ears; open circles) or PBS alone (left ears; closed circles) 1 hour before UV irradiation. Data shown are the mean ± SD (n = 10) of the ear swelling responses at the indicated time points after irradiation. [A]Statistically significant differences ($P < 0.05$) compared with the UV plus PBS group. [B]Statistically significant differences ($P < 0.01$) compared with the UV plus PBS group. [C]Statistically significant differences compared with the UV plus scrambled ODN group ($P < 0.05$). [D]Statistically significant differences compared with the UV plus scrambled ODN group ($P < 0.01$). (**c** and **d**) BALB/c mice received topical application of NF-κB decoy or scrambled ODNs as already described here. Half of these mice were exposed to UV radiation, whereas the other half were sham-irradiated. Ear samples were harvested 4 days after irradiation and subjected to histological examination. Data shown are representative fields after H&E staining (**c**). All specimens (10 ear samples per group) were examined microscopically for ear skin thickness, the number of skin-infiltrating leukocytes, and the number of keratinocyte layers in th e epidermis (**d**). In a different set of experiments, ear specimens were harvested 24 hours after irradiation to determine the impact of NF-κB decoy on sunburn cell formation. Data shown are the mean ± SD (n = 10) of each histological parameter. NT, not tested.

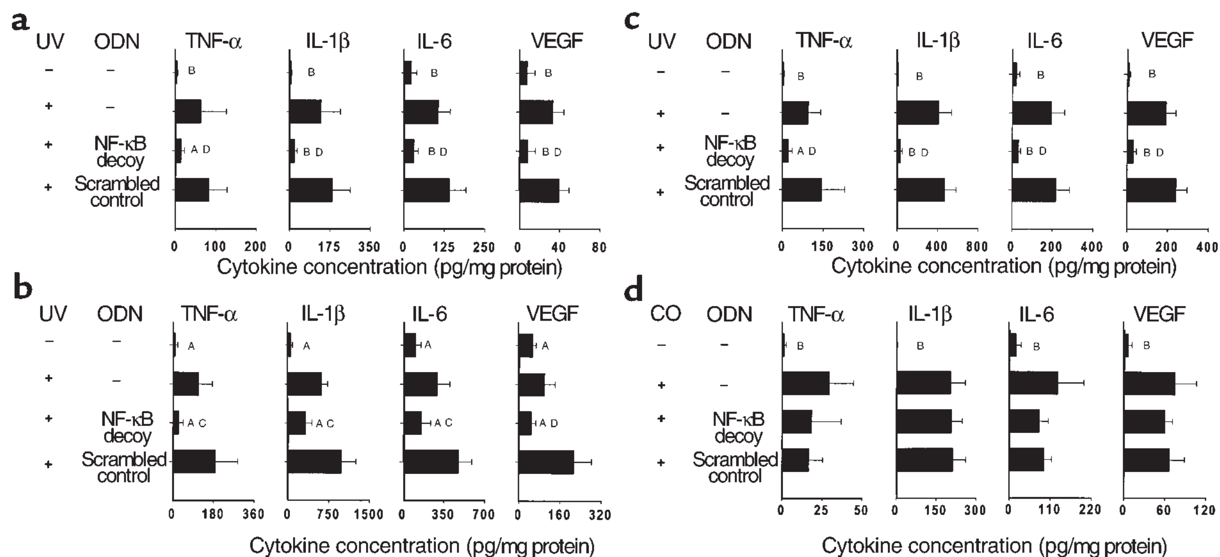

Figure 5
Prevention of UV-induced alteration in local cytokine profiles by NF-κB decoy ODN. (**a** and **b**) BALB/c mice received two intraperitoneal injections of NF-κB decoy or scrambled ODNs. (**c** and **d**) BALB/c mice received topical application of NF-κB decoy or scrambled ODNs. These animals were treated with UV irradiation (**a**–**c**) or topical application of croton oil (CO) onto ear skin (**d**). Ear skin samples harvested 1 day (**a** and **d**) or 4 days (**b** and **c**) after treatment were then examined for the indicated cytokines. Data shown are the mean ± SD (n = 10) of cytokine concentrations, normalized by total protein concentrations in each sample. [A]Statistically significant differences ($P < 0.05$) compared with the UV plus PBS group. [B]Statistically significant differences ($P < 0.01$) compared with the UV plus PBS group. [C]Statistically significant differences compared with the UV plus scrambled ODN group ($P < 0.05$). [D]Statistically significant differences compared with the UV plus scrambled ODN group ($P < 0.01$).

three lineages that were derived from skin, and irradiation concurrently triggered the secretion of VEGF, TNF-α, IL-1β, and IL-6. Second, NF-κB decoy ODNs inhibited significantly UV-induced secretion of each proinflammatory cytokine. Third, systemic, local, or topical administration of NF-κB decoy ODN reduced the severity of experimentally induced sunburn reactions, as assessed by ear swelling, histological changes, and the accumulation of proinflammatory cytokines. On the other hand, NF-κB decoy ODN did not affect noninflammatory changes (Langerhans cell depletion, CPD formation, and sunburn cell formation) that were also observed after radiation, thus, documenting specificity of the pharmacologic activity of NF-κB decoy and the physiological role played by NF-κB–dependent gene transactivation pathways. Finally, topically applied NF-κB decoy ODN resulted in local inhibition of sunburn reactions, without affecting inflammation in nontreated skin sites, with the implication that NF-κB decoy ODN acted primarily on cells that reside in skin. Our conclusion is further supported, albeit indirectly, by the clinical observation that sunburn reactions can be treated successfully by nonsteroidal anti-inflammatory agents (44), which are now known to block NF-κB activation by interfering with IkB kinase-2 (IKK2) activity (45). Moreover, two groups reported independently that mice lacking the IkBα gene developed spontaneously widespread severe dermatitis with leukocyte infiltration, suggesting a pathway through which NF-κB activation directly leads to skin inflammation (46, 47).

In addition to the recent progress in antisense ODN and decoy ODN technologies, an entirely new field of immunomodulatory ODNs has attracted significant attention (48). Several nucleotide sequences or motifs that exert nonspecific immunostimulatory or inhibitory effects have been identified. For example, the 27-bp ODN complementary to a portion of the *rev* region of the HIV gene have been shown to induce polyclonal activation of B cells in vitro (49, 50) and to cause massive splenomegaly and hyper-γ-globulinemia upon in vivo administration (49). The CpG motif, originally identified in bacterial DNA (51), is now known to stimulate both humoral and cellular immune responses via acting on B cells, macrophages, dendritic cells, natural killer cells, and T cells and to control the balance between the Th1- and Th2-type immune responses (52–55). An important question then concerned whether the NF-κB decoy ODNs, indeed, prevented sunburn reactions by blocking NF-κB–dependent gene transactivation pathway. Three lines of evidence support the specificity of our ODN-based approach. First, neither the NF-κB decoy ODNs nor the scrambled control ODNs used in this study contained any of the currently recognized immunomodulatory sequences, including the antisense ODN for the HIV *rev* gene, the conventional CpG motif $(TCG)_n$ or $(CG)_n$ repeats, or palindrome sequences (49–53). Second, the NF-κB decoy ODNs, but not the scrambled control ODNs, blocked NF-κB–mediated transactivation of the Luc gene, without affecting the AP-1–mediated Luc gene expression. Finally, topical application of the NF-κB

decoy ODN prevented UV-induced skin inflammation, without affecting inflammatory responses to skin irritant or allergic compounds. We believe that these observations have excluded the possibility that the NF-κB decoy ODNs prevented sunburn reactions by simply acting as a nonspecific, anti-inflammatory reagent or as a "generic" transcriptional inhibitor.

UV radiation was found, more than a decade ago, to induce the production of TNF-α, IL-1β, and IL-6 by keratinocytes (56–58), and these cytokines are thought to play pathogenic roles in the development of UV-induced cutaneous inflammation (2). More recently, VEGF production by UV-irradiated keratinocytes was proposed to serve as an additional pathogenic event (59, 60). In fact, working with VEGF transgenic mice (keratin 14 promoter), Detmar et al. demonstrated recently that overproduction of VEGF by keratinocytes is sufficient for the development of pathophysiological features characteristic of cutaneous inflammation, including increased vascular density and permeability and enhanced leukocyte rolling and adhesion (61). Thus, it is reasonable to state that UV-induced alterations in the local cytokine milieu are essential to the development of sunburn reactions. Our finding that NF-κB decoy ODNs inhibited both cytokine production and sunburn reactions supports this concept.

Although NF-κB is one of the key transcription factors driving the expression of VEGF, TNF-α, IL-1β, and IL-6 genes (24, 62), it has remained unclear whether UV-induced NF-κB activation indeed plays any functional role in the production of these proinflammatory cytokines after UV irradiation. We have observed that local accumulation of these cytokines in sunburn reactions, but not in contact dermatitis reactions, is inhibited by NF-κB decoy ODNs almost completely. These results indicate that the NF-κB pathway plays an essential role in transducing UV-dependent signals. As described earlier here, UV radiation may lead to NF-κB activation by different mechanisms (e.g., DNA damage, ROI generation, kinase phosphorylation, and ligand-independent receptor signaling), and the present study was not designed to determine the relative contribution of each mechanism. Likewise, it was beyond the scope of this study to determine the role of NF-κB pathway in the development of other UV-induced skin changes (e.g., immunosuppression, accelerated aging, and cancer development). In this regard, topical application of T4 endonuclease immediately after radiation was found to prevent sunburn cell formation and Langerhans cell depletion significantly, without affecting UV-induced skin inflammation (63). Thus, it is conceivable that UV irradiation may cause different types of skin manifestation via different pathways/mechanisms.

In conclusion, the present study has provided the first experimental evidence for the function of NF-κB pathway in the pathogenesis of sunburn reactions. Our data also introduce the concept that sunburn reactions are the culmination of specific inflammatory events transcriptionally triggered by UV-dependent NF-κB activation, instead of a sum of inflammatory changes associated with tissue damage.

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1. Kripke, M.L. 1990. Photoimmunology. *Photochem. Photobiol.* **52**:919–924.
2. Takashima, A., and Bergstresser, P.R. 1996. Impact of UVB radiation on the epidermal cytokine network. *Photochem. Photobiol.* **63**:397–400.
3. Krutmann, J., and Grewe, M. 1995. Involvement of cytokines, DNA damage, and reactive oxygen intermediates in ultraviolet radiation-induced modulation of intercellular adhesion molecule-1 expression. *J. Invest. Dermatol.* **105**(Suppl. 1):67S–70S.
4. Bielenberg, D.R., et al. 1998. Molecular regulation of UVB-induced cutaneous angiogenesis. *J. Invest. Dermatol.* **111**:864–872.
5. Aragane, Y., et al. 1998. Ultraviolet light induces apoptosis via direct activation of CD95 (Fas/APO-1) independently of its ligand CD95L. *J. Cell Biol.* **140**:171–182.
6. Kasibhatia, S., et al. 1998. DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-κB and AP-1. *Mol. Cell.* **1**:543–551.
7. Eller, M.S., Ostrom, K., and Gilchrest, B.A. 1996. DNA damage enhances melanogenesis. *Proc. Natl. Acad. Sci. USA.* **93**:1087–1092.
8. Eller, M.S., Maeda, T., Magnoni, C., Atwal, D., and Gilchrest, B.A. 1997. Enhancement of DNA repair in human skin cells by thymidine dinucleotides: evidence for a p53-mediated mammalian SOS response. *Proc. Natl. Acad. Sci. USA.* **94**:12627–12632.
9. Vink, A.A., et al. 1997. The inhibition of antigen-presenting activity of dendritic cells resulting from UV irradiation of murine skin is restored by in vitro photorepair of cyclobutane pyrimidine dimers. *Proc. Natl. Acad. Sci. USA.* **94**:5255–5260.
10. O'Connor, A., et al. 1996. DNA double strand breaks in epidermal cells cause immune suppression in vivo and cytokine production in vitro. *J. Immunol.* **157**:271–278.
11. Morita, A., et al. 1997. Evidence that singlet oxygen-induced human T helper cell apoptosis is the basic mechanism of ultraviolet-A radiation phototherapy. *J. Exp. Med.* **186**:1763–1768.
12. Scharffetter-Kochanek, K., et al. 1997. UV-induced reactive oxygen species in photocarcinogenesis and photoaging. *Biol. Chem.* **378**:1247–1257.
13. Rosette, C., and Karin, M. 1996. Ultraviolet light and osmotic stress: activation of the JNK cascade through multiple growth factor and cytokine receptors. *Science.* **274**:1194–1197.
14. Simon, M.M., Aragane, Y., Schwarz, A., Luger, T.A., and Schwarz, T. 1994. UVB light induces nuclear factor κB (NFκB) activity independently from chromosomal DNA damage in cell-free cytosolic extracts. *J. Invest. Dermatol.* **102**:422–427.
15. Knebel, A., Rahmsdorf, H.J., and Herrlich, P. 1996. Dephosphorylation of receptor tyrosine kinases as target of regulation by radiation, oxidants or alkylating agents. *EMBO J.* **15**:5314–5325.
16. Derijard, B., et al. 1994. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell.* **76**:1025–1037.
17. Lee, F.S., Hagler, J., Zhijian, J.C., and Maniatis, T. 1997. Activation of the IκB kinase complex by MEKK1, a kinase of the JNK pathway. *Cell.* **88**:213–222.
18. Gilchrest, B.A., Park, H.-Y., Eller, M.S., and Yaar, M. 1996. Mechanisms of ultraviolet light-induced pigmentation. *Photochem. Photobiol.* **63**:1–10.
19. van Dam, H., et al. 1995. ATF-2 is preferentially activated by stress-activated protein kinases to mediate c-*jun* induction in response to genotoxic agents. *EMBO J.* **14**:1798–1811.
20. Grether-Beck, S., et al. 1996. Activation of transcription factor AP-2 mediates UVA radiation- and singlet oxygen-induced expression of the human intercellular adhesion molecule 1 gene. *Proc. Natl. Acad. Sci. USA.* **93**:14586–14591.

21. Iordanov, M., et al. 1997. CREB is activated by UVC through a p38-HOG-1–dependent protein kinase. *EMBO J.* **16**:1009–1022.
22. Schmidt, K.N., Amstad, P., Cerutti, P., and Baeuerle, P.A. 1995. The roles of hydrogen peroxide and superoxide as messengers in the activation of transcription factor NF-κB. *Chem. Biol.* **2**:13–22.
23. Nemoto, S., DiDonato, J.A., and Lin, A. 1998. Coordinate regulation of IκB kinases by mitogen-activated protein kinase kinase kinase 1 and NF-κB-inducing kinase. *Mol. Cell. Biol.* **18**:7336–7343.
24. Barnes, P.J., and Karin, M. 1997. Nuclear factor-κB: a pivotal transcription factor in chronic inflammatory diseases. *N. Engl. J. Med.* **336**:1066–1071.
25. Baeuerle, P.A., and Baltimore, D. 1996. NF-κB: ten years after. *Cell.* **87**:13–20.
26. Sullenger, B.A., Gallardo, H.F., and Gilboa, E. 1990. Overexpression of TAR sequence renders cells resistant to human immunodeficiency virus replication. *Cell.* **63**:601–608.
27. Bielinska, A., Shivdasani, R.A., Zhang, L., and Nabel, G.J. 1990. Regulation of gene expression with double-stranded phosphorothioate oligonucleotides. *Science.* **250**:997–1000.
28. Morishita, R., et al. 1997. In vivo transfection of *cis* element "decoy" against nuclear factor-κB binding site prevents myocardial infarction. *Nat. Med.* **3**:894–899.
29. Ono, S., et al. 1998. Decoy administration of NF-κB into the subarachnoid space for cerebral angiopathy. *Hum. Gene Ther.* **9**:1003–1011.
30. Kawamura, I., et al. 1999. Intratumoral injection of oligonucleotides to the NFκB binding site inhibits cachexia in a mouse tumor model. *Gene Ther.* **6**:91–97.
31. Yuspa, S.H., Hawley-Nelson, P., Koehler, B., and Stanley, J.R. 1980. A survey of transformation markers in differentiating epidermal cell lines in culture. *Cancer Res.* **40**:4694–4703.
32. Xu, S., et al. 1995. Successive generation of antigen-presenting, dendritic cell lines from murine epidermis. *J. Immunol.* **154**:2697–2705.
33. Timares, L., Takashima, A., and Johnston, S.A. 1998. Quantitative analysis of the immunopotency of genetically transfected dendritic cells. *Proc. Natl. Acad. Sci. USA.* **95**:13147–13152.
34. Matsue, H., et al. 1999. Induction of antigen-specific immunosuppression by CD95L cDNA-transfected "killer" dendritic cells. *Nat. Med.* **5**:930–937.
35. Kitajima, T., Ariizumi, K., Bergstresser, P.R., and Takashima, A. 1996. UVB radiation sensitizes a murine epidermal dendritic cell line (XS52) to undergo apoptosis upon antigen presentation to T cells. *J. Immunol.* **157**:3312–3316.
36. Kaminski, M.J., Bergstresser, P.R., and Takashima, A. 1993. In vivo activation of mouse dendritic epidermal T cells in sites of contact dermatitis. *Eur. J. Immunol.* **23**:1715–1718.
37. Kitajima, T., et al. 1996. T cell–mediated terminal maturation of dendritic cells: loss of adhesive and phagocytotic capacities. *J. Immunol.* **157**:2340–2347.
38. Roza, L., van der Wulp, K.J.M., MacFarlane, S.J., Lohman, P.H.M., and Baan, R.A. 1988. Detection of cyclobutane thymine dimers in DNA of human cells with monoclonal antibodies raised against a thymine dimer-containing tetranucleotide. *Photochem. Photobiol.* **48**:627–633.
39. Vink, A.A., et al. 1996. Localization of DNA damage and its role in altered antigen-presenting cell function in ultraviolet- irradiated mice. *J. Exp. Med.* **183**:1491–1500.
40. Elmets, C.A., Bergstresser, P.R., Tigelaar, R.E., Wood, P.J., and Streilein, J.W. 1983. Analysis of the mechanism of unresponsiveness produced by haptens painted on skin exposed to low dose ultraviolet radiation. *J. Exp. Med.* **158**:781–794.
41. Shapiro, V.S., Mollenauer, M.N., Greene, W.C., and Weiss, A. 1996. c-rel regulation of IL-2 gene expression may be mediated through activation of AP-1. *J. Exp. Med.* **184**:1663–1669.
42. Yablonski, D., Kuhne, M.R., Kadlecek, T., and Weiss, A. 1998. Uncoupling of nonreceptor tyrosine kinases from PLC-γ1 in an SLP-76–deficient T cell. *Science.* **281**:413–416.
43. Aberer, W., Romani, N., Elbe, A., and Stingl, G. 1986. Effects of physicochemical agents on murine epidermal Langerhans cells and Thy-1–positive dendritic epidermal cells. *J. Immunol.* **136**:1210–1216.
44. Stern, R.S., and Dobson, T.B. 1985. Ibuprofen in the treatment of UV-B–induced inflammation. *Arch. Dermatol.* **121**:508–512.
45. Yin, M.-J., Yamamoto, Y., and Gaynor, R.B. 1998. The anti-inflammatory agents aspirin and salicylate inhibit the activity of IκB kinase-β. *Nature.* **396**:77–80.
46. Beg, A.A., Sha, W.C., Bronson, R.T., and Baltimore, D. 1995. Constitutive NF-κB activation, enhanced granulopoiesis, and neonatal lethality in IκBα-deficient mice. *Genes Dev.* **9**:2736–2746.
47. Klement, J.F., et al. 1996. I κBα deficiency results in sustained NF-kB response and severe widespread dermatitis in mice. *Mol. Cell. Biol.* **16**:2341–2349.
48. Klinman, D.M., Verthelyi, D., Takeshita, F., and Ishii, K.J. 1999. Immune recognition of foreign DNA: a cure for bioterrorism. *Immunity.* **11**:123–129.
49. Branda, R.F., Moore, A.L., Mathews, L., McCormarck, J.J., and Zon, G. 1993. Immune stimulation by an antisense oligomer complementary to the rev gene of HIV-1. *Biochem. Pharmacol.* **45**:2037–2043.
50. Liang, H., Nishioka, Y., Reich, C.F., Pisetsky, D.S., and Lipsky, P.E. 1996. Activation of human B cells by phosphorothioate oligodeoxynucleotides. *J. Clin. Invest.* **98**:1119–1129.
51. Kreig, A.M., et al. 1995. CpG motifs in bacterial DNA trigger direct B cell activation. *Nature.* **371**:546–549.
52. Iho, S., Yamamoto, T., Takahashi, T., and Yamamoto, S. 1999. Oligodeoxynucleotides containing palindrome sequences with internal 5′-CpG-3′ act directly on human NK and activated T cells to induce IFN-γ production in vitro. *J. Immunol.* **163**:3642–3652.
53. Carson, D.A., and Raz, E. 1997. Oligonucleotide adjuvants for T helper 1 (Th1)-specific vaccination. *J. Exp. Med.* **186**:1621–1622.
54. Hartmann, G., Weiner, G.J., and Kreig, A.M. 1999. CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. *Proc. Natl. Acad. Sci. USA.* **96**:9305–9310.
55. Jakob, T., Walker, P.S., Kreig, A.M., Udey, M.C., and Vogel, J.C. 1998. Activation of cutaneous dendritic cells by CpG-containing oligodeoxynucleotides: a role for dendritic cells in the augmentation of Th1 responses by immunostimulatory DNA. *J. Immunol.* **161**:3042–3049.
56. Köck, A., et al. 1990. Human keratinocytes are a source of tumor necrosis factor α: evidence for synthesis and release upon stimulation and endotoxin or ultraviolet light. *J. Exp. Med.* **172**:1609–1614.
57. Urbanski, A., et al. 1990. Ultraviolet light induces increased circulating interleukin-6 in humans. *J. Invest. Dermatol.* **94**:808–811.
58. Ansel, J.C., Luger, T.A., and Green, I. 1983. The effect of in vitro and in vivo UV irradiation on the production of ETAF activity by human and murine keratinocytes. *J. Invest. Dermatol.* **81**:519–523.
59. Brown, L.F., et al. 1995. Overexpression of vascular permeability factor (VPF/VEGF) and its endothelial cell receptors in delayed hypersensitivity skin reactions. *J. Immunol.* **154**:2801–2807.
60. Brauchle, M., Funk, J.O., Kind, P., and Werner, S. 1996. Ultraviolet B and H_2O_2 are potent inducers of vascular endothelial growth factor expression in cultured keratinocytes. *J. Biol. Chem.* **271**:21793–21797.
61. Detmar, M., et al. 1998. Increased microvascular density and enhanced leukocyte rolling and adhesion in the skin of VEGF transgenic mice. *J. Invest. Dermatol.* **111**:1–6.
62. Chilov, D., et al. 1997. Genomic organization of human and mouse genes for vascular endothelial growth factor C. *J. Biol. Chem.* **272**:25176–25183.
63. Wolf, P., Cox, P., Yarosh, D.B., and Kripke, M.L. 1995. Sunscreens and T4N5 liposomes differ in their ability to protect against ultraviolet-induced sunburn cell formation, alterations of dendritic epidermal cells, and local suppression of contact hypersensitivity. *J. Invest. Dermatol.* **104**:287–292.