The Excimer Lamp Induces Cutaneous Nerve Degeneration and Reduces Scratching in a Dry-Skin Mouse Model

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Epidermal hyperinnervation, which is thought to underlie intractable pruritus, has been observed in patients with atopic dermatitis (AD). The epidermal expression of axonal guidance molecules has been reported to regulate epidermal hyperinnervation. Previously, we showed that the excimer lamp has antihyperinnervative effects in nonpruritic dry-skin model mice, although epidermal expression of axonal guidance molecules was unchanged. Therefore, we investigated the antipruritic effects of excimer lamp irradiation and its mechanism of action. A single irradiation of AD model mice significantly inhibited itch-related behavior 1 day later, following improvement in the dermatitis score. In addition, irradiation of nerve fibers formed by cultured dorsal root ganglion neurons increased bleb formation and decreased nerve fiber expression of nicotinamide mononucleotide adenylyl transferase 2, suggesting degenerative changes in these fibers. We also analyzed whether attaching a cutoff excimer filter (COF) to the lamp, thus decreasing cytotoxic wavelengths, altered hyperinnervation and the production of cyclobutane pyrimidine dimer (CPD), a DNA damage marker, in dry-skin model mice. Irradiation with COF decreased CPD production in keratinocytes, as well as having an antihyperinnervative effect, indicating that the antipruritic effects of excimer lamp irradiation with COF are due to induction of epidermal nerve degeneration and reduced DNA damage.

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INTRODUCTION

Histamine is the best known pruritogen. Although antihistamines are used to treat pruritus, they are relatively ineffective against some dermatological diseases, including atopic dermatitis (AD) (Klein and Clark, 1999; Kabashima, 2013). Such diseases involve an ''itch–scratch cycle,'' in which pruritus induces scratching, which causes intense pruritus and inflammation, sometimes leading to chronic symptoms (Stander *et al.*, 2008). Cure of intractable pruritus requires therapeutic strategies to interrupt this cycle.

Many inducers and mediators of intractable pruritus have been associated with barrier disruption and inflammation (Kabashima, 2013). Epidermal hyperinnervation is one factor underlying intractable pruritus in both humans and animals with AD (Urashima and Mihara, 1998; Tominaga and Takamori, 2013). Expression of axonal guidance molecules in the epidermis has been reported to regulate epidermal hyperinnervation, with dominance of nerve elongation factors (e.g., nerve growth factor; NGF) over nerve repulsion factors (e.g., semaphorin 3A), resulting in the elongation of nerve fibers into the epidermis (Tominaga and Takamori, 2013).

Various therapeutic modalities focusing on nerve fibers in the skin have been described recently. Psolaren ultraviolet A therapy normalized the expression of NGF and semaphorin 3A and the distribution of epidermal nerve fibers in the skin of AD patients, ameliorating pruritus (Tominaga *et al.*, 2009). Treatment with anti-NGF agents and supplementation with semaphorin 3A significantly inhibited epidermal hyperinnervation and scratching behavior, improving dermatitis in NC/Nga mice with AD-like symptoms, compared with untreated mice (Takano *et al.*, 2005, 2007; Yamaguchi *et al.*, 2008; Negi *et al.*, 2012). These findings indicate that epidermal hyperinnervation is a factor of intractable itch, and that blocking epidermal hyperinnervation may be effective in the treatment of intractable pruritus.

Using nonpruritic dry-skin model mice, we showed that a monochromatic 308-nm xenon chloride (XeCl) excimer lamp has antihyperinnervative effects without altering the epidermal expression of axonal guidance molecules (Kamo *et al.*, 2011b). Therefore, the antipruritic effect and mechanisms underlying the antihyperinnervative effect of the excimer lamp are still

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Abbreviations: AD, atopic dermatitis; AXIS, axon investigation system; COF, cutoff excimer filter; CPD, cyclobutane pyrimidine dimer; DRG, dorsal root ganglion; NGF, nerve growth factor

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unclear. The excimer lamp is a valid therapeutic option for the treatment of some dermatoses, such as AD and psoriasis (Aubin *et al.*, 2005; Nistico *et al.*, 2008). Moreover, irradiation with a 308-nm XeCl excimer laser suppressed pruritus in AD patients (Baltas *et al.*, 2006).

Here, we assessed the antipruritic effects of the excimer lamp on pruritic AD model mice, as well as the mechanism underlying the antihyperinnervative effects of excimer lamp exposure on cultured dorsal root ganglion (DRG) neurons. Attachment of the excimer lamp to a cutoff excimer filter (COF), reducing exposure to unwanted wavelengths, was shown to reduce the production of cyclobutane pyrimidine dimers (CPDs) in Jurkat T cells and a human skin equivalent model (Kobayashi *et al.*, 2009). Therefore, we examined the effects of excimer lamp irradiation on epidermal hyperinnervation and the risk of DNA damage to epidermal keratinocytes in the mouse dry-skin model.

RESULTS

Effects of excimer lamp irradiation on scratching behavior

AD-like lesions were induced in the skin of NC/Nga mice by repeated topical applications of *Dermatophagoides farinae* body (Dfb) ointment for 3 weeks, increasing the number of scratching bouts. As two sessions of excimer laser irradiation promptly ameliorated the pruritus (Baltas *et al.*, 2006), we examined the effects of one or two sessions of excimer lamp irradiation at 250 mJ cm^{-2}, with the COF attached on scratching behavior and Dfb-induced dermatitis (Figure 1a, experiment 1). The number of scratching bouts decreased significantly even after a single session (Figure 1b), although the difference in dermatitis score between the two groups was small (Figure 1c). We also examined the dose-dependent effects of a single irradiation over time (Figure 1a, experiment 2). Dermatitis score tended to improve in a dose-dependent manner in irradiated animals, although the difference between control and excimer lamp–irradiated groups was not significant (Figure 1d and e). The densities of intraepidermal nerve fibers (Figure 1f) and nerve fibers penetrating into the epidermis (Figure 1g) tended to decrease in the irradiated group, compared with controls (Supplementary Figure S1 online).

Effects of excimer lamp irradiation on cultured DRG nerve fibers

To assess the direct effects of excimer lamp irradiation on nerve fibers, we used an axon investigation system (AXIS), which can separate nerve fibers from cell bodies (Figure 2a and b). DRG neurons were plated onto silica glass slides, through which 308-nm light can pass, and irradiated with the excimer lamp from beneath, with a shield for cell bodies (Figure 2a). After 10 days in culture, nerve fibers were irradiated and the effects of irradiation were assessed at the indicated times (Figure 2c).

The degenerating nerve fibers showed increased bleb formation (Figure 2d, arrows) (Coleman, 2005). Irradiation increased the number of blebs per nerve fiber in all experimental groups (Figure 2e), with significant differences between the number of blebs at 2.5 and 120 hours. The shielded areas in all experimental groups showed no evidence of degeneration, such as increased numbers of blebs in nerve fibers and detachment of cell bodies (data not shown). There was no change in the number of blebs in the control group. The numbers of blebs following irradiation at 100 and 250 mJ cm^{-2} were reduced by 0.8-fold ($P=0.045$) and 0.6-fold ($P=0.021$), respectively, in the presence compared with the absence of the COF (Figure 2e). However, after irradiation with the COF at both 100 and 250 mJ cm^{-2}, the number of blebs increased significantly from 2.5 to 120 hours (Figure 2e).

Nicotinamide mononucleotide adenylyl transferase 2 (Nmnat2) is an essential factor for neuron survival. This protein is involved in anterograde transport and is depleted in distal stumps of injured neurons before the formation of blebs (Wang *et al.*, 2012). Excimer lamp irradiation at 250 mJ cm^{-2} with COF reduced Nmnat2 expression in nerve fibers (Figure 3a) compared with control (Figure 3b). Decreased Nmnat2 expression was observed before changes in cytoskeletal organization, as shown by staining for neuronal class III β-tubulin with TuJ1 antibody (Figure 3a).

Effects of COF on epidermal hyperinnervation

COF can cut out unwanted wavelengths and prevent the formation of CPDs, a product of DNA damage, in cultured T cells and a human skin equivalent model (Kobayashi *et al.*, 2009). Therefore, we examined the effects of the COF on epidermal keratinocytes and hyperinnervation using neighboring areas in dry-skin model mice, with the level of barrier disruption assessed by transepidermal water loss (TEWL) (data not shown). In this model, peak hyperinnervation occurs 48 hours after acetone treatment. Mice were therefore irradiated with the excimer lamp 24 hours after acetone treatment, and mouse skin samples were obtained 24 hours after irradiation (Figure 4a).

Excimer lamp irradiation induced CPD formation in the epidermis of all irradiated groups (Figure 4b). The ratio of CPD-positive (CPD$^+$) to 4′,6-diamidino-2-phenylindole hydrochloride–stained nuclei was >60% in all experimental groups (Figure 4c). Compared with mice irradiated without COF, those irradiated with 250 mJ cm^{-2}, but not with 100 mJ cm^{-2}, showed a significant reduction in the fluorescence intensity of epidermal CPD$^+$ nuclei (Figure 4d).

In contrast, irradiation had marked antihyperinnervative effects, with little difference between irradiated groups (Figure 4e). Measurements of the numbers of intraepidermal nerve fibers (Figure 4f) and of nerve fibers penetrating into the epidermis (Figure 4g) showed a >50% antihyperinnervative effect in all experimental groups (Figure 4f and g). This antihyperinnervative effect was dose dependent in both the presence and absence of the COF (Figure 4f and g).

DISCUSSION

The present study showed that excimer lamp irradiation significantly suppressed the scratching behavior of atopic-NC/Nga mice, even after one irradiation session, following improvement of the dermatitis score (Figure 1). Excimer lamp irradiation to nerve fibers of cultured DRG neurons promoted degeneration of these fibers, as indicated by the increased number of blebs and the decreased expression of Nmnat2 (Figures 2 and 3). As treatments that suppress

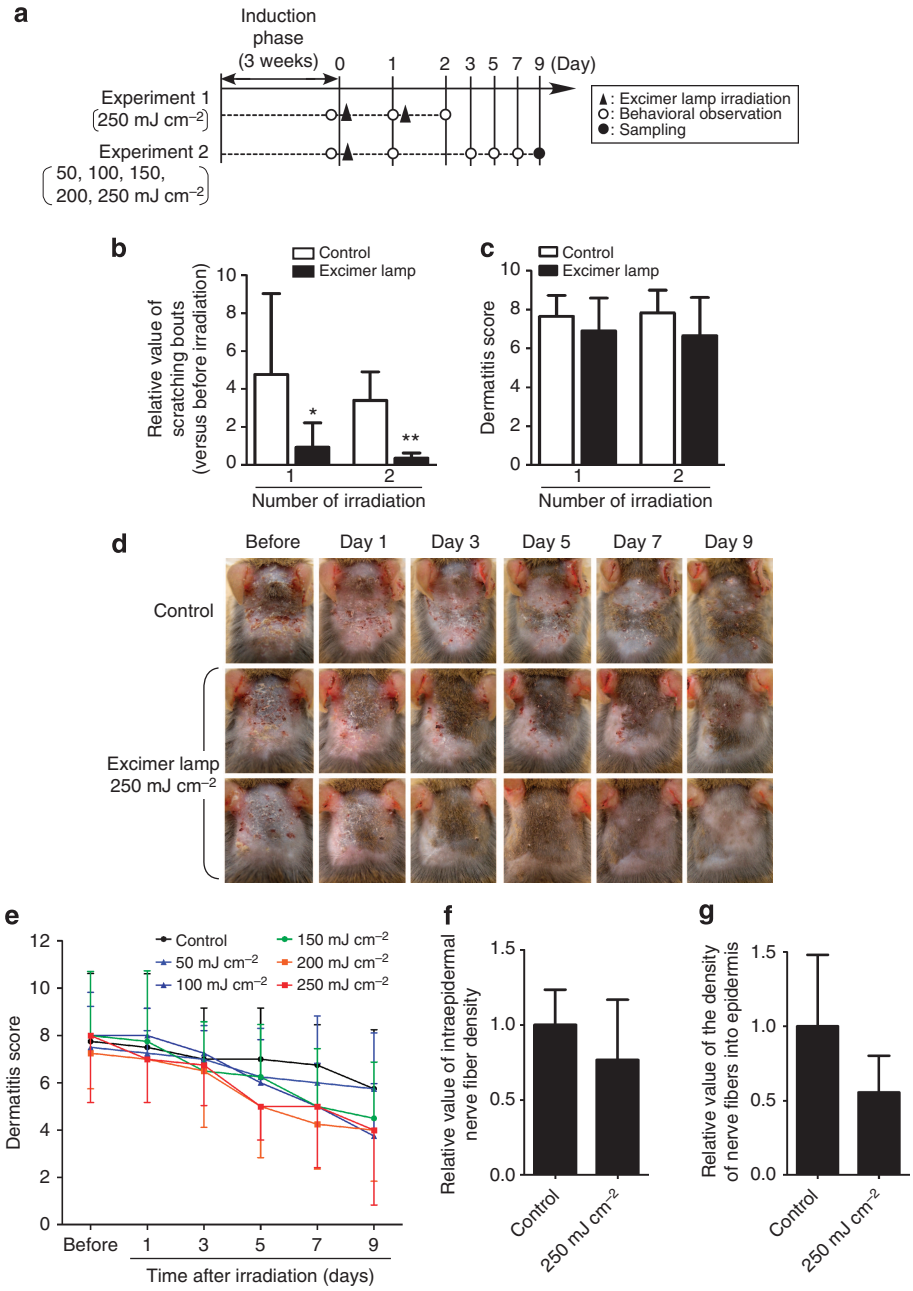

Figure 1. Effects of excimer lamp irradiation on scratching behavior. (**a**) Experimental scheme. Following the induction of atopic dermatitis (AD)-like dermatitis, experiments 1 and 2 were conducted to examine the effects of excimer lamp irradiation for 2 and 9 days, respectively. (**b**) Effects of excimer lamp irradiation on scratching bouts 1 day after one or two sessions. Even a single irradiation with the excimer lamp significantly decreased the number of scratching bouts compared with the control. *$P<0.05$, **$P<0.01$ versus control at each point. (**c**) Effects of excimer lamp irradiation on dermatitis score 1 day after one or two sessions. There was no significant difference between control and excimer lamp–irradiated groups. (**d**) Effects of excimer lamp irradiation on AD-like lesions. Excimer lamp irradiation tended to improve dermatitis. (**e**) Effects of excimer lamp single irradiation on dermatitis score over time. A tendency to improve the dermatitis score was observed in a dose-dependent manner, although the difference between control and excimer lamp–irradiated groups was not significant. (**f**, **g**) Effects of excimer lamp single irradiation on the densities of intraepidermal nerve fibers (**f**) and nerve fibers penetrating into the epidermis (**g**). Although there was no significant difference, the densities of intraepidermal nerve fibers and nerve fibers penetrating into the epidermis tended to decrease in the irradiated group. All values represent the means ± SD of 4–12 animals.

epidermal hyperinnervation at least partly contribute to attenuating pruritus (Aubin *et al.*, 2005; Tominaga *et al.*, 2009), our findings suggest that the antipruritic effects of excimer lamp irradiation were due in part to nerve degeneration.

In our *in vivo* study using NC/Nga mice, excimer lamp monotherapy markedly decreased the number of scratching bouts, even after a single session (Figure 1b). Patients with AD essentially scratch the sites of dermatitis (Ebata *et al.*, 1999). Removing the claws of NC/Nga mice, thus preventing

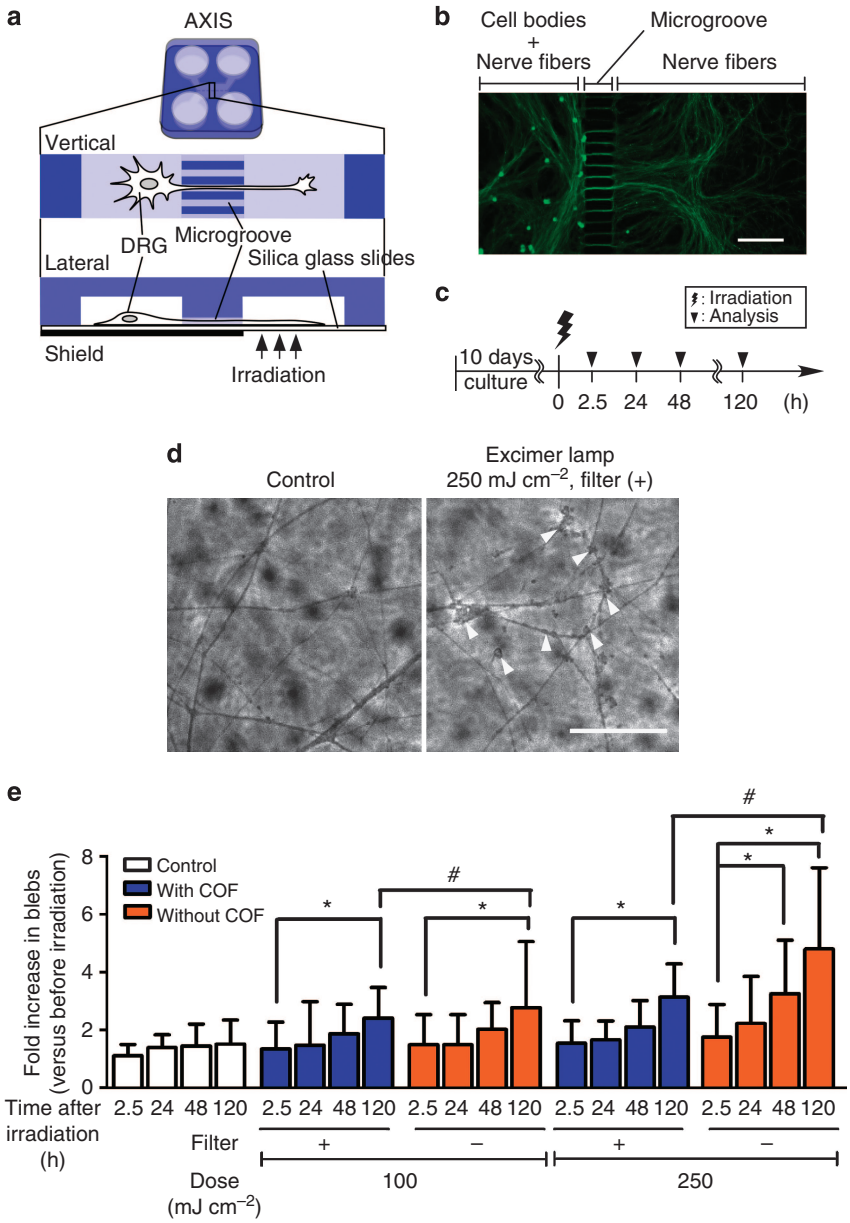

Figure 2. Effects of excimer lamp irradiation on bleb formation in nerve fibers. (**a**) Dorsal root ganglion (DRG) fibers separated from cell bodies were mounted onto silica glass slides and irradiated from beneath, while cell bodies were shielded. (**b**) Immunofluorescence staining with anti-TuJ1 antibody showing the separated DRG fibers. Scale bars = 250 μm. (**c**) After culture for 10 days, the effects of irradiation were reassessed at the indicated time points. (**d**) Morphological changes in the nerve fibers 120 hours after irradiation at 250 mJ cm^{-2}. Arrows indicate irradiation-induced blebs. Scale bars = 50 μm. (**e**) Numbers of blebs at various time points after irradiation. *P<0.05 compared with 2.5 hours, $^{\#}P$<0.05 compared with the absence of the cutoff excimer filter (COF). All values represent the means ± SD of three experiments.

scratching, successfully inhibited the induction and progression of dermatitis (Hashimoto *et al.*, 2004). These results suggest that reduction or prevention of scratching, or lesions resulting from scratching, is important in the treatment of AD. Therefore, the decreased number of scratching bouts observed after a single session of excimer lamp irradiation and its ability to interrupt the itch–scratch cycle suggests that this treatment method may be clinically useful.

In addition, we found that excimer lamp irradiation of atopic-NC/Nga mice slightly improved the dermatitis score (Figure 1e) and reduced the density of both intraepidermal nerve fibers and nerve fibers penetrating into the epidermis (Figure 1f and g). Previously, we reported that excimer lamp irradiation had no effect on the epidermal expression of axonal guidance molecules, such as NGF and semaphorin 3A (Kamo *et al.*, 2011b). Given that excimer lamp does not normalize the imbalance, it is possible that the antihyperinnervative effect may be transient and nerve fibers in the dermis may later penetrate into the epidermis again. We previously reported that topical application of emollients, such as heparinoid cream, normalized the epidermal imbalance in the expression of axonal guidance molecules in dry-skin

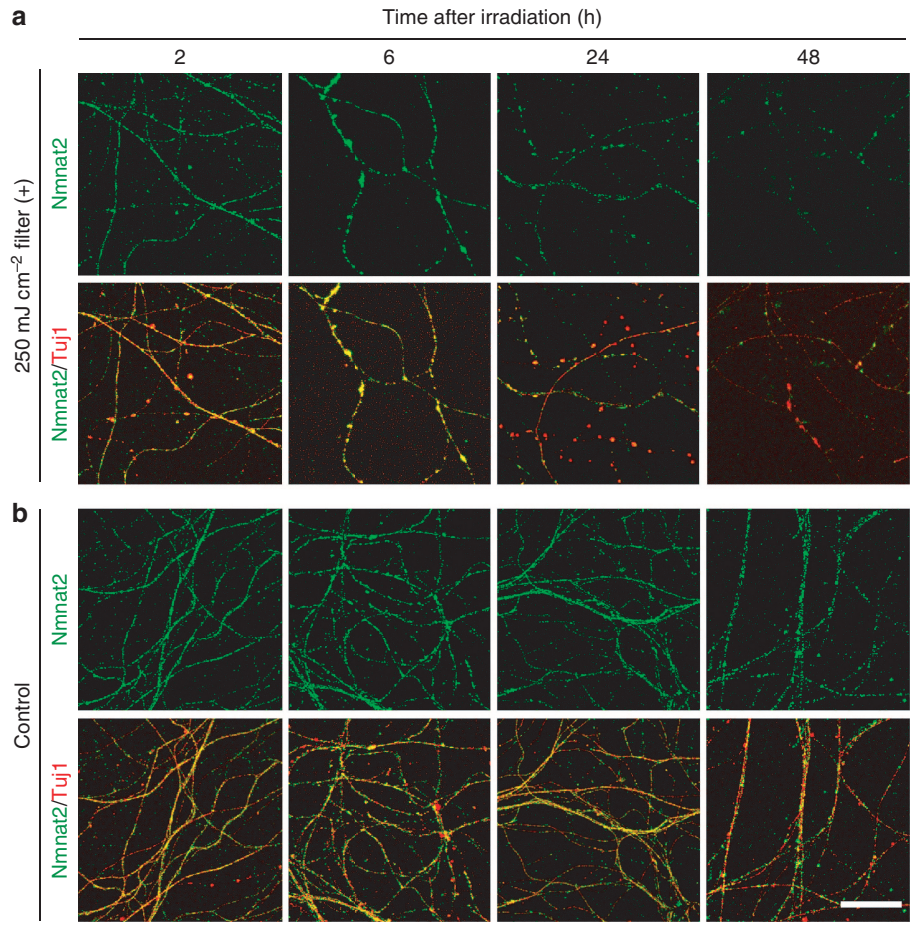

Figure 3. Effects of excimer lamp irradiation on Nmnat2 expression in nerve fibers. Double labeling of Nmnat2 (green) and TuJ1 (red) in nerve fibers irradiated with the excimer lamp at 250 mJ cm^{-2} in the presence of the cutoff excimer filter (**a**) and in control nerve fibers (**b**). Irradiation resulted in a gradual decrease in Nmnat2 expression over time compared with controls. Scale bars = 20 μm.

model mice (Kamo *et al.*, 2011a). Thus, the findings presented here suggest that a combination of excimer lamp irradiation and emollients may be effective in the treatment of patients with AD.

In our *in vitro* experiment using the AXIS culture system, excimer lamp ultraviolet irradiation of nerve fibers induced a degenerative change (Figures 2e and 3). Previous studies have shown that ultraviolet radiation impairs axonal transport in neurons (Schnapp and Reese, 1989). We also found that the level of Nmnat2, which is involved in anterograde axonal transport (Wang *et al.*, 2012), was decreased in the nerve fibers of cultured DRG neurons after irradiation by excimer lamp (Figure 3). Partial or complete blockade of axonal transport by mechanical injury also induces the degeneration of nerve fibers (Coleman, 2005; Gilley and Coleman, 2010). Taken together, although the molecular mechanism of neurodegenerative effects of excimer lamp remains unknown, the irradiation may affect axonal transport, probably leading to nerve fiber degeneration.

Excimer lamp irradiation using COF was previously shown to reduce DNA damage in Jurkat T cells and keratinocytes in human skin equivalent model (Kobayashi *et al.*, 2009). We showed in this study that excimer lamp irradiation with COF had antihyperinnervative effects and decreased CPD production in epidermal keratinocytes (Figure 4). Thus, these findings show that the excimer lamp used with a COF is both safe and effective for the treatment of itch in dry skin.

Excimer lamp irradiation may be a cause of neuropathy, owing to an absence of innervation. Alterations in intraepidermal nerve density are likely to be associated with the severity of neuropathy in patients with diabetes (Tesfaye *et al.*, 2010). Although irradiation in this study was limited to the skin, and there are clear differences between systemic neuropathy in patients with diabetes and localized neuropathy resulting from excimer lamp irradiation, further studies are needed to assess the risk of neuropathy after irradiation.

In conclusion, our findings suggest that excimer lamp irradiation may have antipruritic effects through the induction of epidermal nerve degeneration. These hyperinnervative effects were observed even when COF decreased damage to DNA.

MATERIALS AND METHODS

Antibodies and reagents

The primary antibodies used in this study were mouse anti-Nmnat2 (1:100 dilution; Abcam, Cambridge, MA), rabbit TuJ1 antibody anti-neuronal class III β-tubulin (1:200 dilution; Cell Signaling

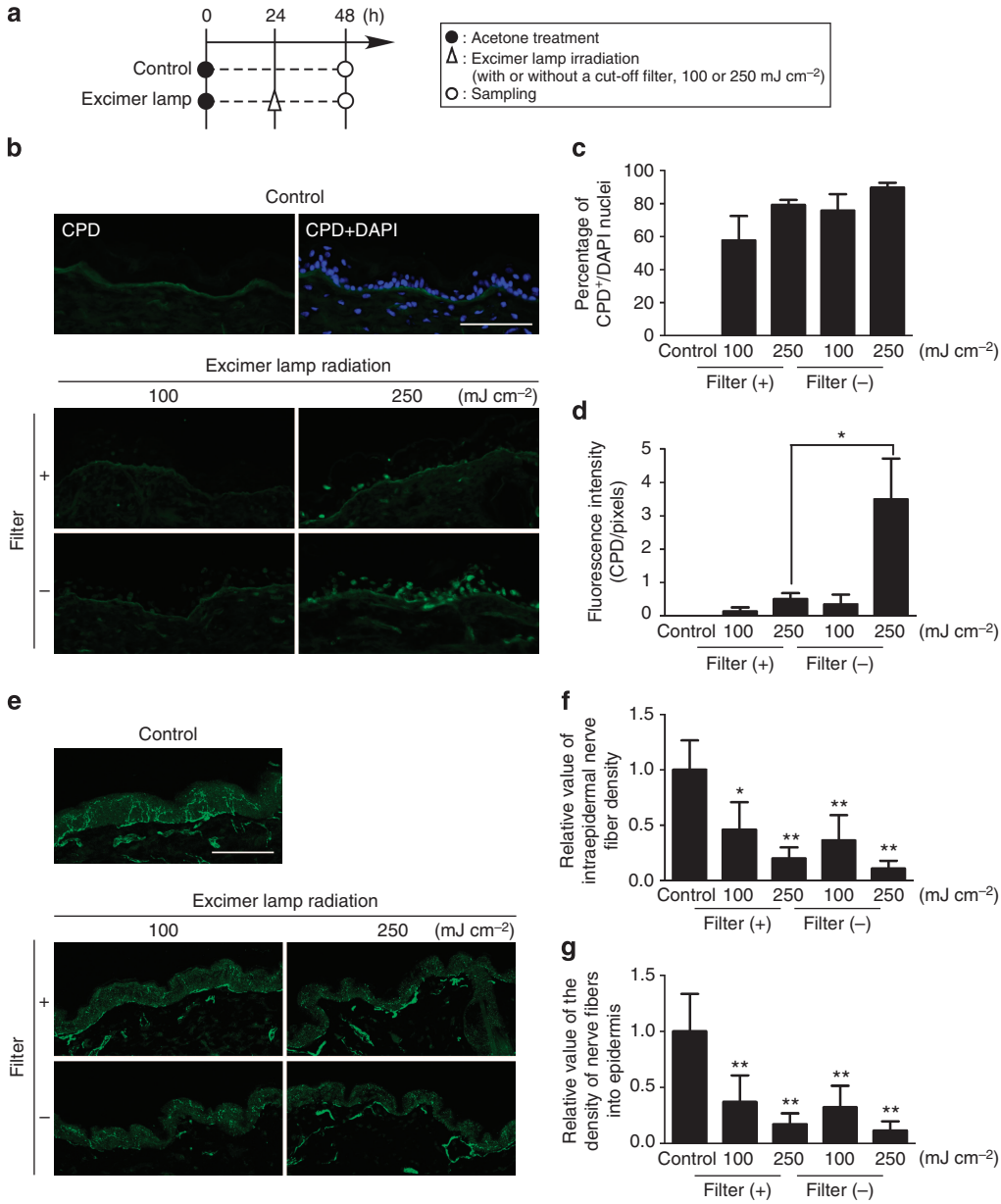

Figure 4. Effects of cutoff excimer filter (COF) on epidermal hyperinnervation. (**a**) Experimental scheme. Dry-skin model mice were irradiated 24 hours after acetone treatment, and skin samples were obtained 24 hours later. (**b**) Distribution of cyclobutane pyrimidine dimer-positive (CPD^+) nuclei. Scale bars = 50 μm. (**c**) Ratio of CPD^+ to 4′,6-diamidino-2-phenylindole hydrochloride (DAPI)-stained nuclei, showing increases in all irradiated groups compared with nonirradiated controls. (**d**) COF suppression of CPD fluorescence intensity in the epidermis. $*P<0.01$. All values represent the means ± SD of five experiments. (**e**) Distribution of intraepidermal nerve fibers in the skin, showing that excimer lamp irradiation inhibited epidermal hyperinnervation. Scale bars = 50 μm. (**f**, **g**) Suppression by COF of intraepidermal nerve density (**f**) and the density of nerve fibers elongating into the epidermis (**g**). $*P<0.01$, $**P<0.001$. All values represent the means ± SD of five experiments.

Technology, Beverly, MA), rabbit anti-protein gene product 9.5 (PGP9.5, 1:400 dilution; LifeSpan BioSciences, Seattle, WA), and rabbit anti-CPD (1:1500 dilution; Cosmo Bio, Tokyo, Japan). Secondary antibodies conjugated with Alexa Fluor dye (1:300 dilution) were purchased from Molecular Probes (Eugene, OR). N-2 supplement was from Invitrogen (Carlsbad, CA), *Dermatophagoides farinae* body (Dfb) ointment (Biostir-AD) was from Biostir (Kobe, Japan), and BSA was from Sigma-Aldrich (St Louis, MO). Normal donkey serum was purchased from Chemicon (Temecula, CA), normal goat serum from Immuno-Biological Laboratories (Gunma, Japan), and Vectashield mounting medium from Vector Laboratories (Burlingame, CA).

Animals

Male ICR mice (10 weeks old; SLC Japan, Shizuoka, Japan) and NC/Nga mice (10 weeks old; Oriental Yeast, Tokyo, Japan) were maintained in the experimental animal facility of Juntendo University Graduate School of Medicine under a 12-hour light:12-hour dark cycle at a regulated temperature of 22–24 °C, with food and tap water provided *ad libitum*. Care and handling of all animals conformed to

the NIH guidelines for animal research, and all animal procedures were approved by the Institutional Animal Care and Use Committee of Juntendo University Graduate School of Medicine.

Dermatophagoides farinae body (Dfb)–induced AD-like model

Dermatitis was induced in NC/Nga mice as described (Yamamoto *et al.*, 2007). Briefly, on day 0, the mice were anesthetized with sevoflurane, the rostral part of the back was clipped with an electric shaver, and residual hair was depilated using hair removal cream. Ointment containing 100 mg of Dfb was applied to the shaved area. Before the second and subsequent applications, growing hair on the rostral skin was removed with an electric shaver and 150 μl of 4% SDS was applied for barrier disruption 2 hours before the application of Dfb ointment. This procedure was repeated twice weekly for 3 weeks. To confirm the lesional skin condition, dermatitis score and the number of scratching bouts were measured. For excimer lamp irradiation, mice with scores >5 were used.

Evaluation of dermatitis score

The severity of AD-like dorsal skin lesions was assessed according to four symptoms: erythema/hemorrhage, scarring/dryness, edema, and excoriation/erosion. Each symptom was graded from 0 to 3 (none, 0; mild, 1; moderate, 2; severe, 3). Dermatitis score was defined as the sum of the individual scores, and it ranged from 0 to 12 (Suto *et al.*, 1999).

Measurement of scratching behavior

Scratching behavior was measured as described (Kuraishi *et al.*, 1995). Mice were placed individually in acrylic cages composed of four cells ($13 \times 9 \times 35$ cm). A camcorder (Model HDR-SR11; Sony, Tokyo, Japan) was positioned above the cages to record the animals' behavior. After an acclimation period of at least 1 hour, the animals' behavior was recorded on video for at least 2 hours with no experimenters present in the observation room. Scratching behavior was assessed by replaying each video. An incidence of scratching behavior was defined as raising and lowering a leg.

Culture of DRG neurons using AXIS

Neonatal rat DRG neurons were purchased from Cambrex (Walkersville, MD) and maintained in serum-free DMEM/F12 medium (Invitrogen) supplemented with 20 ng ml^{-1} NGF, 0.5% N-2 supplement, 87.5 ng ml^{-1} 5-fluoro-2′-deoxyuridine, 37.5 ng ml^{-1} uridine, 50 U ml^{-1} penicillin, and 50 μg ml^{-1} streptomycin at 37 °C in an atmosphere of 5% CO_2.

An AXIS microfluidic chamber (Millipore, Billerica, MA) was used according to the manufacturer's protocols. Briefly, silica glass slides were coated with poly-D-lysine and laminin (both from Sigma), and a chamber was placed onto each coated slide. Approximately 2×10^4 DRG neurons were plated onto one side of the channel in the chamber and cultured for 10 days before irradiation.

Preparation of dry-skin model

The protocol for cutaneous barrier disruption has been described (Tominaga *et al.*, 2007). Briefly, the hair over the rostral part of the back of ICR mice was shaved at least 3 days before acetone treatment, and each shaved area was treated with acetone-soaked cotton balls for 5 minutes. Barrier disruption was assessed 1 hour later by measuring transepidermal water loss for 30 seconds using a Tewameter TM210 (Courage & Khazawa, Cologne, Germany).

Excimer lamp irradiation

A TheraBeam UV308 XeCl excimer lamp (Ushio, Tokyo, Japan) was used for irradiation. The attached excimer filter blocked wavelengths below 297 nm (Kobayashi *et al.*, 2009). The intensity, measured with a UIT-250/UVD-S313 (Ushio) was 15 mW cm^{-2}. Nerve fibers from DRG cells cultured using AXIS were irradiated from beneath the silica glass slides. Mice were anesthetized before irradiation. The minimal erythema dose 24 hours after excimer lamp irradiation was over 450 mJ cm^{-2}. The irradiation experiments were conducted with doses lower than the minimal erythema dose.

Immunocytochemistry

Cultured cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 minutes, permeabilized with 1% Triton X-100 in phosphate-buffered saline (PBS, pH 7.4) for 10 minutes, and incubated with 5% normal goat serum in PBS containing 1% BSA for 1 hour. Following overnight incubation at 4 °C with primary antibodies against Nmnat2 and TuJ1, the cultured neurons were incubated with Alexa Fluor dye–conjugated secondary antibodies for 1 hour and mounted in Vectashield mounting medium (Vector Laboratories). Immunoreactivity was viewed with a confocal laser-scanning microscope (DMIRE2; Leica Microsystems, Wetzlar, Germany).

Immunohistochemistry

Skin samples were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 4 hours, washed with PBS, and immersed successively in PBS solutions containing 10, 15, and 20% sucrose. The skin samples were embedded in an optimal cutting temperature compound (Sakura Finetek Japan, Tokyo, Japan), frozen in liquid nitrogen, and cut into cryosections (20-μm-thick for PGP9.5 staining and 8-μm-thick for CPD staining) using a CM1850 cryostat (Leica Microsystems). The sections were mounted onto silane-coated glass slides. For immunofluorescence staining of PGP9.5, the sections were incubated in PBS containing 5% normal donkey serum and 2% BSA, followed by overnight incubation at 4 °C with an antibody against PGP9.5. The sections were washed with PBS and incubated with Alexa Fluor dye–conjugated secondary antibody for 1 hour at room temperature. For immunofluorescence staining of CPD, the sections were incubated at 37 °C for 7 minutes with proteinase K (50 mg ml^{-1} stock; Roche, Mannheim, Germany) diluted 1:1,000 in proteinase K buffer (50 mM Tris, 1 mM ethylenediaminetetraacetic acid, pH 8.5, 0.5% Tween 20) and incubated in 70 mM NaOH in 70% ethanol to denature the nuclear DNA. Following washing in PBS and blocking with 5% normal donkey serum and 2% BSA, as described above, the sections were incubated overnight at 4 °C with an antibody against CPD. After washing with PBS, the sections were incubated with Alexa Fluor dye–conjugated F(ab′)2 fragments for 1 hour at room temperature, washed with PBS, and mounted in Vectashield mounting medium with 4′,6-diamidino-2-phenylindole hydrochloride. Immunoreactivity was viewed with a confocal laser-scanning microscope (DMIRE2; Leica Microsystems) or a fluorescence microscope (BIOREVO BZ-9000; Keyence, Osaka, Japan).

Semiquantification of nerve degeneration

Bright-field images of three sites from each experiment were captured at the indicated times. The numbers of blebs and nerve fibers in 10 boxes

of 100 × 100 μm from each site were hand-counted by two researchers (AK and YK). Blebs more than twice the width of the associated nerve fibers were counted. The ratio of blebs to nerve fibers in each box was calculated and compared with the ratio before irradiation. All values are reported as the means ± SD of three experiments.

Semiquantification of epidermal nerve fibers

Nine skin specimens from each mouse were stained with anti-PGP9.5 antibody. Using a confocal microscope, 0.9-μm-thick optical sections were scanned through the *z*-plane of the stained specimens, and the images were reconstructed in three dimensions using Leica Confocal Software (Leica Microsystems). The numbers of PGP9.5-immunoreactive nerve fibers penetrating into and within the epidermis were hand-counted by two researchers (MT and UK) in a blinded manner. In these analyses, a typical line structure with a minimum length of 12.5 μm was counted as one fiber, with counting based on intraepidermal nerve fiber counting rules (Lauria *et al.*, 2005). The area of the epidermis was also evaluated in these images, and the nerve fiber density was calculated. All values are reported as the means ± SD of five animals.

Semiquantification of CPD formation

Nine skin specimens from each mouse were stained with anti-CPD antibody. The numbers of CPD^+ and 4′,6-diamidino-2-phenylindole hydrochloride-stained nuclei in the epidermis were hand-counted by two researchers (MT and UK) in a blinded manner. The ratios of CPD^+ to 4′,6-diamidino-2-phenylindole hydrochloride-stained nuclei were calculated. The fluorescence intensity of the epidermis was also evaluated using the ImageJ image analysis program (1.46r; National Institutes of Health, Bethesda, MD). Exposure and acquisition settings were fixed such that no signal saturation occurred. All values are reported as the means ± SD of five animals.

Statistical analysis

Statistical analyses were performed using the two-tailed Student's *t* test and one-way or two-way ANOVA with Bonferroni's multiple comparison test using the Prism 5 software (GraphPad Software, La Jolla, CA). In all analyses, $P<0.05$ was taken to indicate statistical significance.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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