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Vitamin D3 and Neurofibromatosis Type 1

Juichiro Nakayama

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Abstract

Vitamin D3 (VD3) and its analogs have been shown to inhibit growth of various cell types found in neurofibromas and pigmented lesions of patients with neurofibromatosis type 1 (NF1). Excimer light irradiation at 150–300 mJ/cm^2 in combination with VD3 efficiently inhibited growth of cultured fibroblasts, mast cells, Schwann cells, and melanocytes. Long-term whole body irradiation with narrowband ultraviolet B (UVB) in patients with NF1 significantly increased serum levels of VD3, which was accompanied by a brightening of generalized skin hyperpigmentation. Irradiation with either laser or intense pulsed-radio frequency in combination with topical application of VD3 analogs yielded moderate to fair improvement of café-au-lait macules, small pigmented spots, and skin-fold freckling in NF1 patients. Thus, topical or systemic application of VD3 or one of its analogs may provide beneficial effects to treat skin lesions for patients with NF1.

Keywords: neurofibromatosis type 1, café-au-lait macule, neurofibroma, vitamin D3, narrowband UVB

1. Introduction

Neurofibromatosis type 1 (NF1), also known as von Recklinghausen's disease, is an autosomal dominant disorder affecting approximately 1 in 3500 people. Common to all ethnic origins, NF1 appears to occur in similar proportions for all individuals without sex or ethnic specificity. Riccardi [1] classified heterogenous neurofibromatosis disorders into eight categories. Hallmarks of NF1, the classic type of neurofibromatosis, include café-au-lait macules (CALMs), and multiple benign cutaneous neurofibromas (NFs), comprising dermal, subcutaneous, and plexiform types. Clinical diagnosis is based on the presence of two or more of the following findings: six or more CALMs (largest diameter >0.5 cm in prepubertal individuals, or >1.5 cm

in postpubertal individuals); two or more NFs of any type, or one plexiform NF; axillary freckling; optic glioma; two or more Lisch nodules within the iris; a distinctive osseous lesion; or first-degree relative diagnosed with NF1 according to the preceding criteria [2]. While significant advances in understanding both the pathoetiology and genetics of NF1 have been made in the last decade, no therapeutic modalities are currently available for NF1 patients; although several studies are currently examining various agents specifically directed at plexiform NF, such as clinical trials for sirolimus and imatinib mesylate [3].

We have previously investigated the effects of vitamin D3 (VD3) and its analogs on skin lesions of patients with NF1 and observed inhibited growth of fibroblasts primarily isolated from NFs (previously called fibroblastic cells) [4]. We next performed in vivo experiments in which we found topical application of a VD3 analog onto a CALM grafted to nude mouse skin inhibited uptake of bromodeoxyuridine into cells on the basal layer of grafted epidermis [5]. In addition, cell density of NF tissues subcutaneously grafted onto the skin of nude mice decreased significantly with direct local injection of a VD3 analog [6]. Consequently, as an initial human study, we evaluated the effect of 1 month of VD3 analog application twice a day to an NF1 patient's large pigmented plaque. We observed moderate improvement of the applied macule, including a remarkable decrease in intensity of Fontana Masson staining in the epidermal basal layer [6]. We extended our study to examine whether VD3 or its analogs inhibit growth of Schwann cells and mast cells isolated from primary NFs. We also investigated the capacity of these agents to inhibit growth of human epidermal melanocytes. Our results indicated that with the exception of Schwann cells, all cell types examined were inhibited by VD3 and its analogs in vitro.

From a molecular aspect, the NF1 gene encodes a Ras GTPase-activating protein called neurofibromin; mutations in this gene affect Ras-mitogen-activated protein kinase (MAPK) signaling [7]. Inhibition of this signaling, also known as the Ras/Raf/MEK/ERK or MEK pathway, has also been shown to be efficacious in treating human NF [8]. Therefore, we examined inhibitory effects of a mechanistic target of rapamycin (mTOR) inhibitor (rapamycin) and a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor (lovastatin) [9] on growth of Schwann cells isolated from primary NFs. Our results demonstrated that these agents significantly inhibited Schwann cell growth in vitro.

We examined the potential of other therapeutic modalities to be combined with VD3 for even greater improvement of skin lesions in patients with NF1. Previous reports indicate narrow-band ultraviolet B (NB-UVB) irradiation directly induces conversion of vitamin D to VD3 in various cell types including human keratinocytes [10, 11]; thus, we analyzed whether long-term whole body NB-UVB irradiation increased serum levels of VD3 in NF1 patients. We also observed the effects of irradiation on patient skin lesions by photographing treatment areas before and after irradiation procedures. Our results showed that 6 months of irradiation or more increased patient VD3 serum levels significantly. This increase was accompanied by changes in the patients' skin color including lightening of generalized hyperpigmentation.

It has been established that laser treatments have little effect on pigmented skin lesions, such as CALMs or small freckling, of patients with NF1. Instead, we investigated the effects of laser or intense pulsed-radio frequency (IPL-RF) irradiation in combination with topical application

of VD3 analogs. In one young patient, treatment with Q-switched Ruby laser irradiation in combination with a topical VD3 analog treatment resulted in almost complete disappearance of a CALM located on the nose. A similar therapy combining IPL-RF irradiation with topical VD3 application was very effective at diminishing multiple small pigmented freckles on the face and body of another patient. Herein, our recent investigative results concerning the effects of VD3 and its analogs on NF1 are described in greater detail.

2. Methods

2.1. Cell culture

Primary fibroblasts isolated from NFs of patients with NF1 were cultured as previously described [4]. Human epidermal melanocytes (Melanocel 1; Kurabo, Japan) were cultured according to the manufacturer's instructions. Isolation of Schwann cells and mast cells was performed as previously described [12]. Briefly, NF pieces were dissociated in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific K.K., Yokohama, Japan) containing collagenase (Life Technologies, Carlsbad, CA) and dispase (Roche Diagnostics, Basel, Switzerland), and then cells were resuspended in DMEM containing fetal calf serum, antibiotics, 3-iso-butyl-L-methlxanthine (Sigma, St Louis, MO), β-heregulin (Wako, Japan), forskolin (Sigma) and insulin (Sigma). Isolated Schwann cells were seeded onto culture flasks coated with poly-L-Lysine (Sigma) and laminin (Life Technologies). To isolate mast cells, NF pieces were incubated in a digestion buffer containing Hank's Balanced Salt Solution (Life Technologies), collagenase (Worthington Biochemical, Lakewood, NJ), hyaluronidase (Worthington Biochemical), and DNase (Sigma). Cells were layered over 75% Percoll® (Sigma) and centrifuged, and then nucleated cells were collected from the buffer-Percoll interface. Percoll-enriched gradient cells were suspended in AIM-V® Medium (Life Technologies) containing human stem cell factor (PeproTech, Rocky Hill, NJ). The purity of Schwann or mast cell populations was assessed with S-100 or toluidine blue staining, respectively, and a purity grade of more than 90% was confirmed for both cell-type isolations. Cells were used after 2–3 passages.

2.2. Treatment of cells with VD3 and its analogs with or without excimer light

Fibroblasts from NFs and human epidermal melanocytes were seeded at a density of 2×10^4 cells/ml onto 35-mm cell culture dishes for 2 days. Next, cells were treated for 3 days with 10^{-9}–10^{-7} M calcitriol (VD3, 1α,25-dihydroxyvitamin D3; Enzo Life Sciences, Farmingdale, NY), tacalcitol [1,24-dyhydroxyvitamin D3; Teijin, Japan], or 22-oxacalcitriol (OCT; 1α,25-dihydroxy-22-oxavitamin D3; Chugai Pharmaceuticals, Japan). Cells were trypsinized and then dissociated cells were collected by centrifugation and counted by Coulter counter. Melanocytes treated with agents were also labeled with 1 μCi of ^{3}[H]-thymidine (^{3}[H]-TdR); incorporation into melanocytes was counted by liquid scintillation counter, as previously described for counting fibroblasts isolated from NFs [4]. Both cell types were exposed to excimer light (308 nm) at doses of 150–300 mJ/cm^2 in the presence of calcitriol and tacalcitol and were then

cultured for 3 days. Excimer light (308-nm UVB) was used to irradiate culture dishes instead of 308 nm of UVB because the tip of the excimer light was small and handy and it allowed for precise irradiation of an expected dose. In the following experiments, isolated primary fibroblasts (three patients), mast cells (five patients), and Schwann cells (six patients) were cultured as described above and then treated with 0.1 μM calcitriol or tacalcitol for 3 days. Cells irradiated with excimer light in the presence or absence of agents were cultured for 3 days after irradiation. Floating mast cells were centrifuged before counting. Mean counts of triplicate measurements were determined.

2.3. Treatment of cells with rapamycin and/or lovastatin

Isolated primary Schwann cells and fibroblasts from NFs were seeded at a density of 1.0–2.0 × 10^4 cells/ml onto 35-mm culture dishes. After 2 days, cells were treated with rapamycin (0.1–100 nM), lovastatin (0.1–10 μM), or a combination of both for 3 days. Number of cells per dish was evaluated by Coulter counter and mean counts of triplicate measurements were determined for each dish, as previously described [13].

2.4. NB-UVB irradiation

In the dermatology outpatient clinic at Fukuoka University Hospital, NF1 patients who had complained of itching or painful sensation were administered whole body NB-UVB irradiation (312 ± 2 nm) at doses of 0.2–0.5 J/cm^2 once weekly or biweekly (every 2 weeks). Approximately, half of these patients presented with complicating atopic dermatitis. During irradiation, serum VD3 levels of patients who had received NB-UVB irradiation for more than 18 months were measured and compared with those of patients without any treatment. Next, nine patients who had not received any previous treatment were irradiated with NB-UVB for 6 months and serum VD3 levels were measured before and after irradiation for a kinetic study. Patients not showing an increase in serum VD3 levels, even after 6 months of irradiation, were further irradiated for more than a year to study whether additional irradiation increased serum VD3 levels. Special Laboratory References (Tokyo, Japan) measured serum VD3 concentration of patient serum samples using a VD3 Radioimmunoassay Kit (Immunodiagnostic Systems, UK) in accordance with the manufacturer's instructions. As a preliminary clinical investigation, we also examined whether increases in serum (and possibly skin) VD3 levels brought about by long-term NB-UVB irradiation bestowed any benefit to NF1 patient skin lesions [10, 11]. We photographed the examined areas before and after irradiation, especially focusing on generalized pigmentation of the skin.

## 2.5. IPL-RF or laser irradiation with or without topical VD3 analogs

We investigated combinations of topical VD3 analogs with various forms of irradiation including NB-UVB, IPL-RF (Aurora; Syneron Medical, Israel), Q-switched Ruby (JMEC, Japan), and neodymium-doped yttrium-aluminum-garnet (Nd:YAG) laser (MedLight C6; Cynosure, Westford, MA) treatments. IPL-RF irradiation was performed according to previously described methods [14, 15]. Laser toning procedures using a Q-switched Nd:YAG laser were carried out during 5–10 sessions over 1- to 2-week interval with 2.5 J/cm^2 at 5 Hz. Clinical

improvement of skin lesions was determined by photographs or using a colorimeter (Crystaleye; Olympus, Japan) before and after treatments.

All clinical studies were performed with prior approval from an ethics committee at Fukuoka University Hospital, and all enrolled patients agreed to the studies and provided informed consent.

3. Results

We previously reported that VD3 and its analogs inhibited growth of primary fibroblasts isolated from NFs, whereby around 30–50% of fibroblast growth inhibition was observed with 0.1 μM calcitriol, tacalcitol, or OCT [4]. This inhibition rate almost corresponded to that of isolated primary normal human fibroblasts. A decreased number of fibroblasts was accompanied by 50–70% inhibition of ^{3}[H]-TdR uptake, observed as early as 2–4 days after addition of agents to culture medium [4]. We also tested whether growth of human epidermal melanocytes was inhibited by VD3 and its analogs and found similar levels of cell growth inhibition and ^{3}[H]-TdR uptake (**Figure 1**).
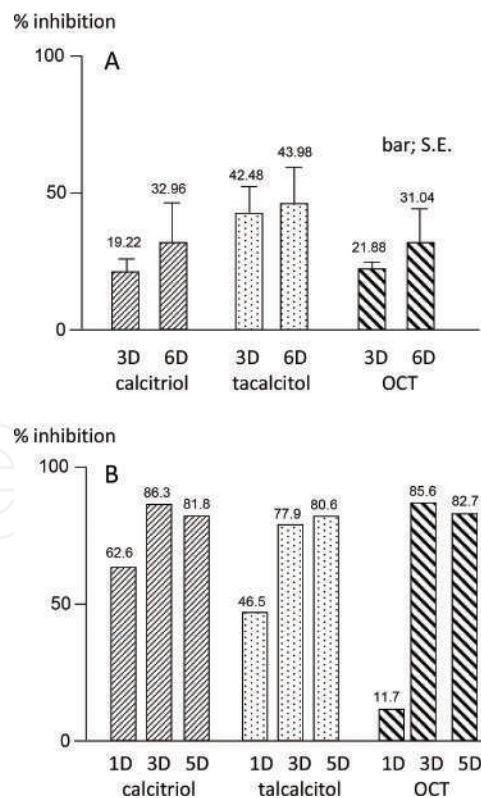

Figure 1. Growth inhibition of human melanocytes with addition of calcitriol, tacalcitol, or OCT. After treatment, number of cells was counted with a Coulter counter and ^{3}[H]-TdR uptake was measured by liquid scintillation. Percent inhibition of cell growth (A) and ^{3}[H]-TdR uptake (B) are shown.

We observed growth inhibition in fibroblasts from NFs and human melanocytes was increased by 10^{-9} to 10^{-7} M calcitriol or tacalcitol in combination with excimer light irradiation. Indeed, calcitriol and tacalcitol inhibited growth of both cell types in a dose-dependent manner, and this inhibition was further augmented by around 20–30% with 150–300 mJ/cm² of excimer light irradiation (**Figure 2**). Recent progress in cell culture techniques allowed us to isolate and culture Schwann and mast cells from NFs with a purity of more than 90%, as described in the Methods section. Primary fibroblasts, mast cells, and Schwann cells were isolated from NFs and examined for how they responded to 0.1 μM calcitriol or tacalcitol and/or excimer light irradiation. Growth of mast cells was inhibited by 16–20% with 0.1 μM calcitriol or tacalcitol; whereas with excimer light irradiation at a dose of 300 mJ/cm², this value rose with statistical significance to 26% (almost the same as that of fibroblasts). Also, a small additive inhibition was observed with 0.1 μM calcitriol or tacalcitol and excimer light irradiation at a dose of 300 mJ/cm² in mast cells. With regard to Schwann cells, neither VD3 nor tacalcitol had an effect on their growth, an unexpected result. Excimer light irradiation inhibited growth of Schwann cells by approximately 38% in a statistically significant manner (**Figure 3**).
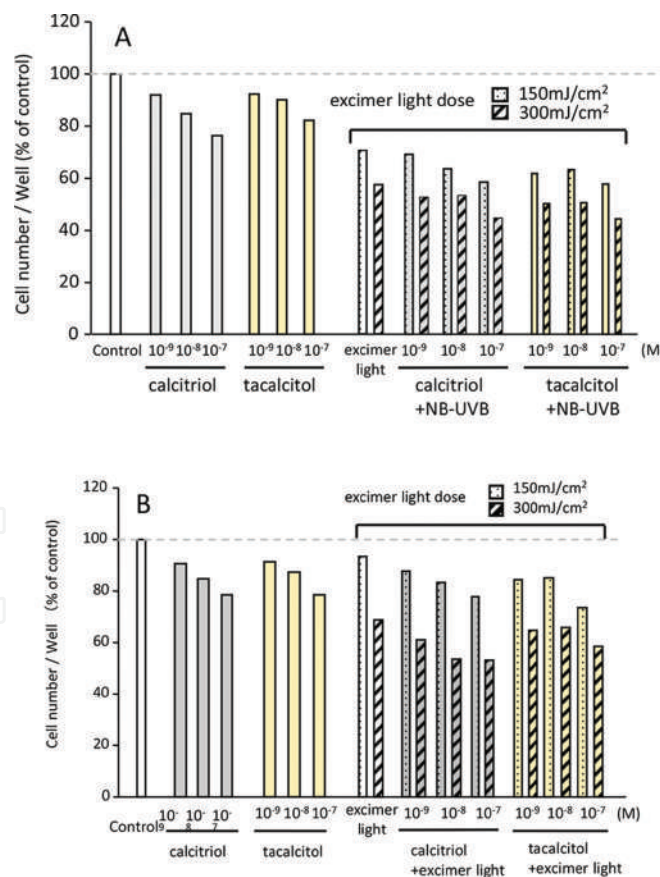

Figure 2. Inhibition of cell growth by calcitriol or tacalcitol in combination with excimer light irradiation. Fibroblasts isolated from NFs and human melanocytes were irradiated with excimer light in the presence or absence of calcitriol or tacalcitol, and number of cells per dish was counted. Data for fibroblasts (A) and melanocytes (B) are shown.
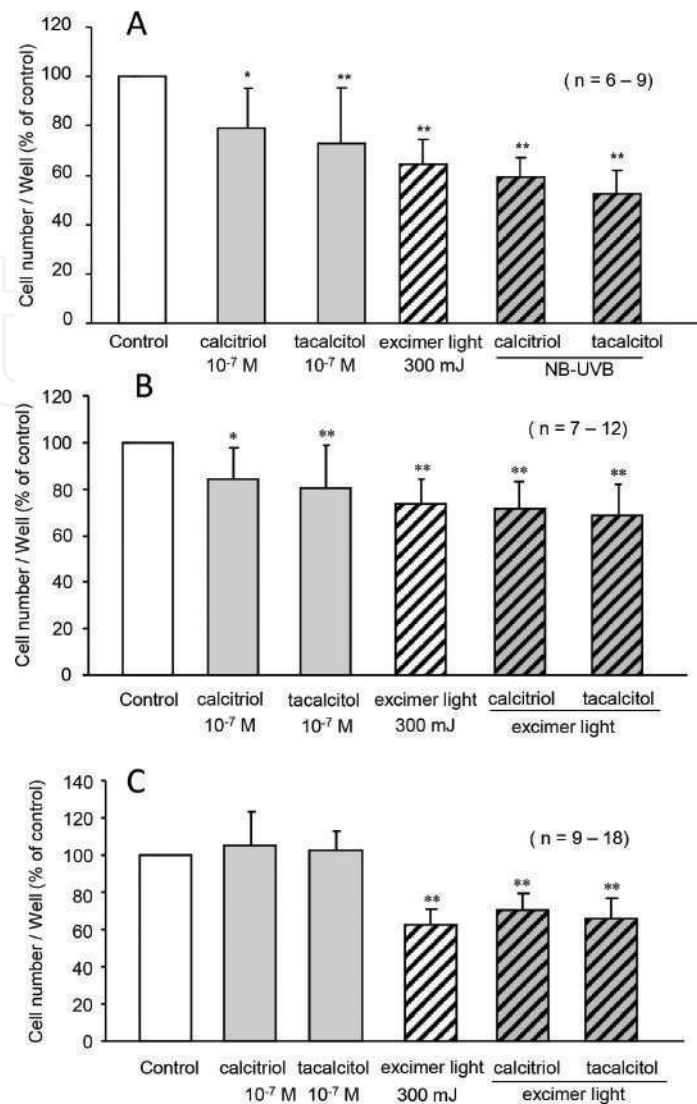
Figure 3. Inhibitory effects of excimer light irradiation with or without calcitriol or tacalcitol treatment on the growth of fibroblasts (A), mast cells (B), and Schwann cells (C). *$P < 0.05$ and **$P < 0.01$ vs. control cells. Values represent mean ± standard deviation.

As we did not see any effect of VD3 or its analogs on Schwann cells, we investigated whether rapamycin or lovastatin inhibited growth of these cells using isolated primary fibroblasts as a control. Growth of primary Schwann cells isolated from NFs was inhibited by 0.1–10 nM rapamycin or 0.1–10 μM lovastatin in a dose-dependent manner. Furthermore, combination of 0.3 nM rapamycin with 1 μM lovastatin resulted in a considerable additive inhibitory effect on the growth of Schwann cells. Growth inhibition rates for fibroblasts with either rapamycin or lovastatin were consistently less than those of Schwann cells (**Figure 4**). We also examined whether a combination of rapamycin and/or lovastatin with VD3 augmented growth inhibitory effects on Schwann cells and fibroblasts. Our experimental results showed that addition of 0.1 μM calcitriol slightly diminished the growth inhibitory effect of 0.3 nM of rapamycin and 1 μM of lovastatin. In contrast, the combination of these agents showed additive effects on fibroblast growth inhibition (data not shown).
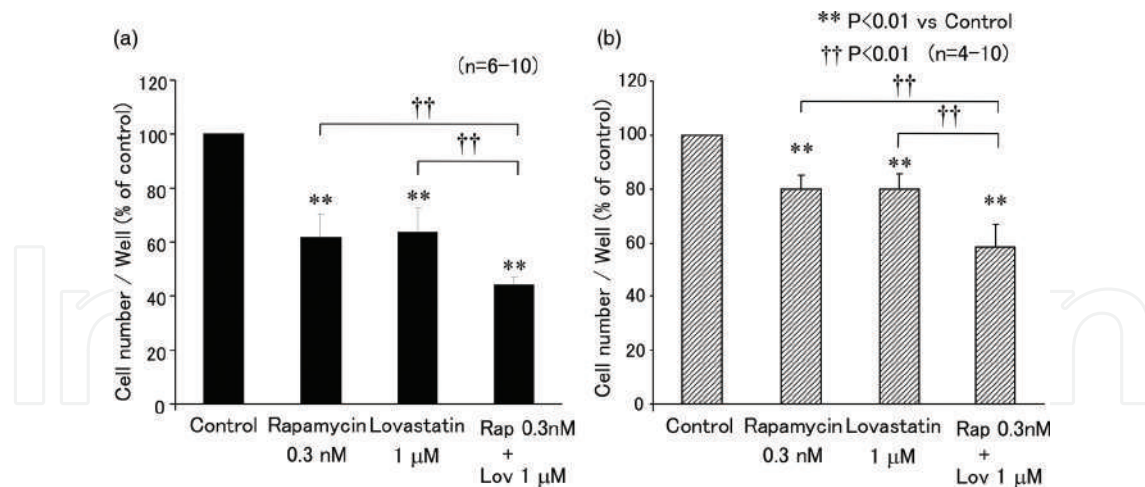
Figure 4. Significant growth inhibition of Schwann cells(A) and fibroblasts(B) isolated from NFs by 0.3 nM rapamycin and/or 1 μM lovastatin.

We investigated whether long-term whole body NB-UVB irradiation increased serum VD3 levels in patients with NF1 and found that patients irradiated once weekly or biweekly (0.2–0.5 J/cm^2 dose per irradiation) for at least 18 months had significantly increased serum VD3 levels compared with patients receiving no treatment (**Figure 5**). A kinetic analysis of this increase subsequently carried out in nine patients revealed that 6 months of irradiation was enough to significantly increase serum VD3 levels (**Figure 6**). Finally, we also observed that significant increases in serum VD3 levels occurred when irradiation with NB-UVB was continued for more than 1 year in patients previously demonstrating low serum VD3 levels after an initial 6 months of irradiation (**Figure 7**).
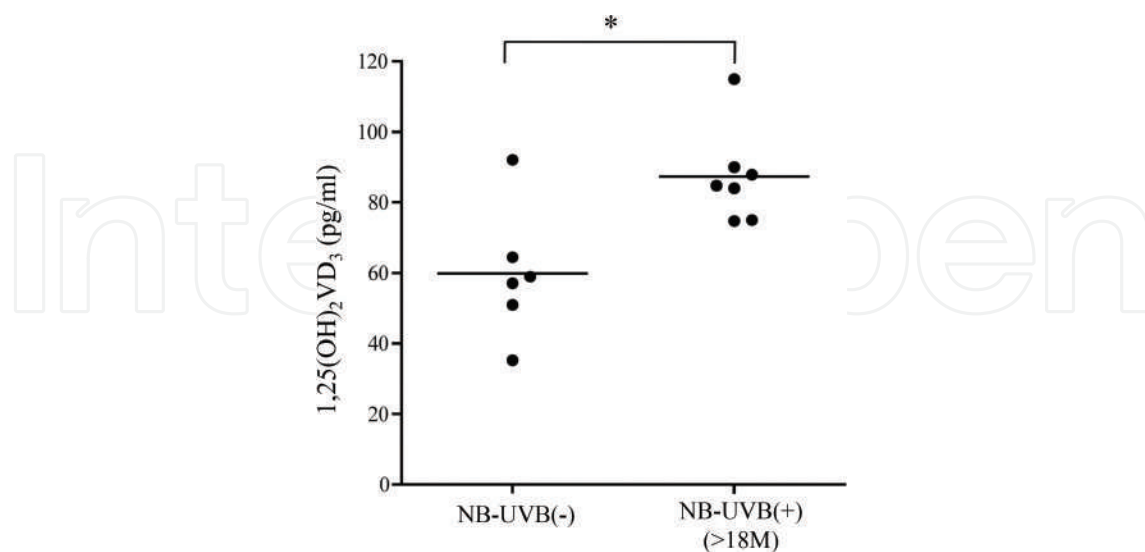
Figure 5. Increase in serum VD3 levels in NF1 patients irradiated with NB-UVB at doses of 0.2–0.5 J/cm^2 once weekly or biweekly for more than 18 months; levels compared with untreated patients (*$P < 0.05$).
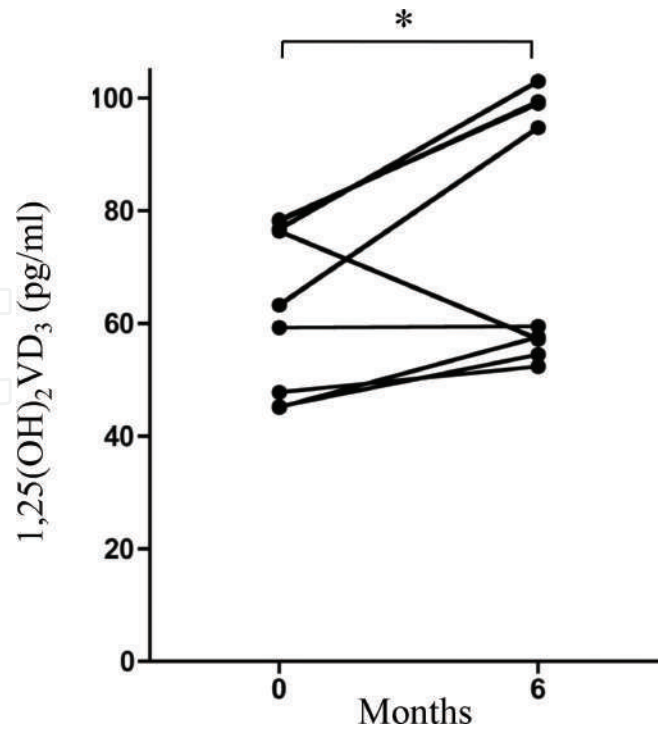
Figure 6. Time course of serum VD3 levels in NF1 patients irradiated with NB-UVB. Serum VD3 levels of patients before and after 6 months of NB-UVB irradiation were measured (*P < 0.05).
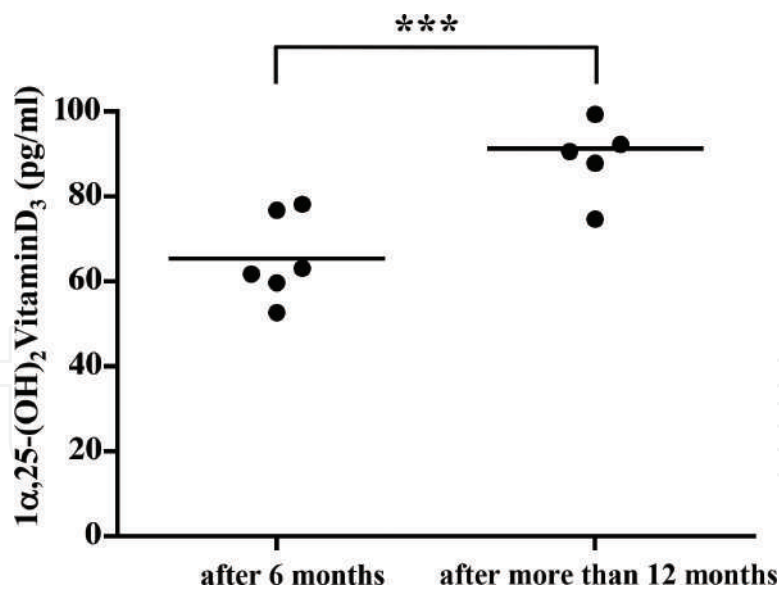
Figure 7. Increase in serum VD3 levels in NF1 patients who had not shown an increase in VD3 levels, even after an initial 6 months of NB-UVB irradiation, after continuing irradiation treatment for more than 1 year thereafter. Statistical analysis was performed using a paired t-test (***P < 0.001).

We examined whether CALMs, small pigmented freckles, or generalized hyperpigmentation improved in 10 patients after more than 6 months of NB-UVB irradiation (once weekly or

biweekly). Using photography, we observed general hyperpigmentation in most patients receiving NB-UVB irradiation became brighter; although effects on CALMs and small pigmented spots were not apparent within this period (**Figure 8**). A questionnaire completed by the patients indicated that 80% (8/10) believed they had either excellent or fair improvement of pigmented skin lesions, and all of the patients were satisfied with the results of NB-UVB irradiation.
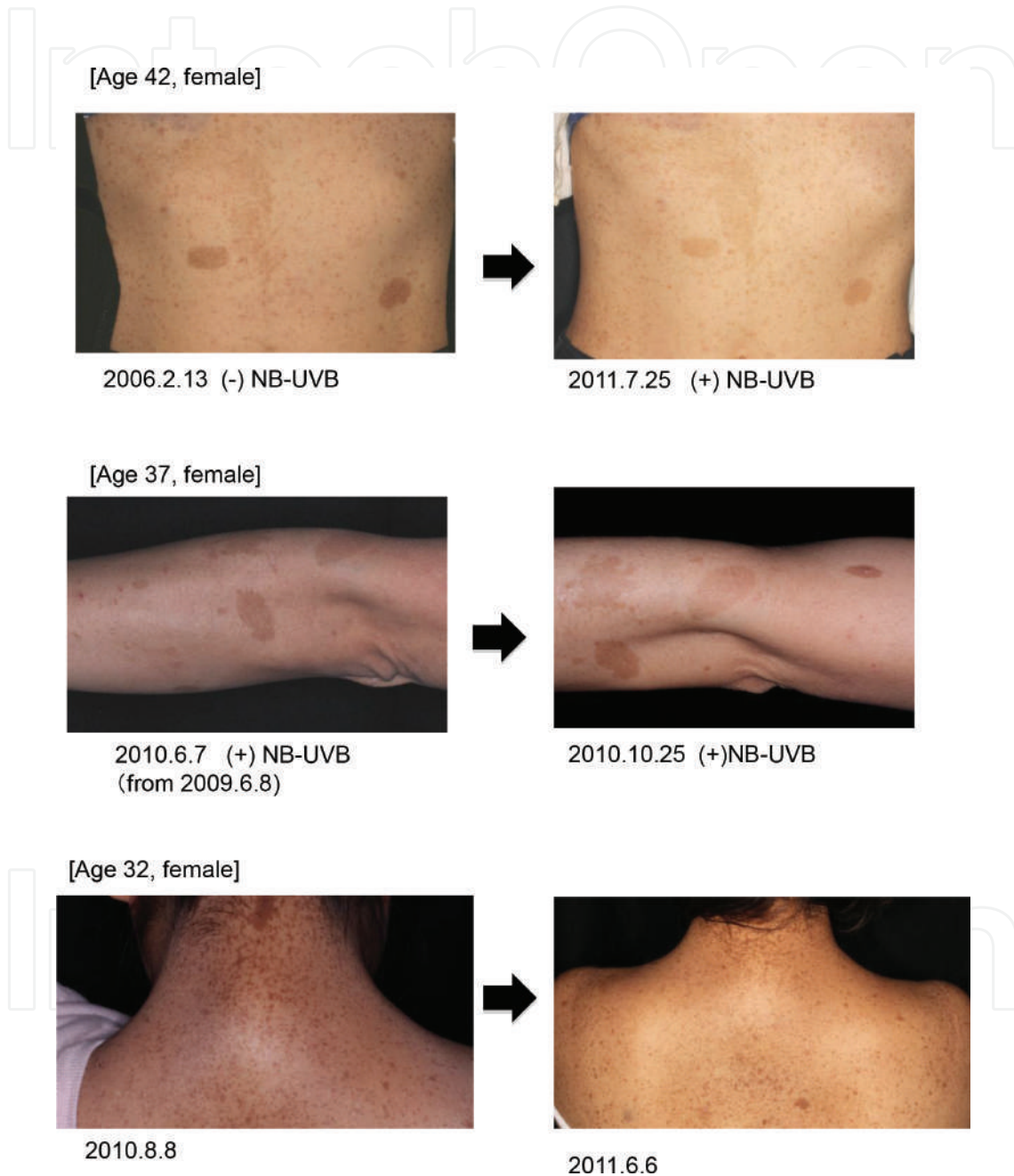
Figure 8. Photographs of three typical cases, showing lightened skin color of generalized hyperpigmentation after NB-UVB irradiation at doses of 0.3–0.5 J/cm² weekly or biweekly for more than 6 months.

Next, the effects of combining IPL-RF irradiation with topical OCT application on CALMs and multiple small pigmented spots were investigated. We IPL-RF irradiated multiple pigmented

spots on the body of a 27-year-old female patient. Multiple pigmented spots received either OCT only or IPL-RF irradiation only for comparison to the area treated with both IPL-RF and OCT. Remarkable improvement of multiple pigmented spots was observed after six IPL-RF irradiation procedures and continuous topical OCT application over a 4-month period (**Figure 9**). In addition, the area treated only with IPL-RF irradiation showed similar improvements to the combination treatment area; however, areas treated with OCT only or OCT plus IPL-RF exhibited increased lightness of skin appearance, as measured by colorimeter [14]. We then treated an additional eight NF1 patients with this combination therapy and observed a moderate to good response with regard to lightening of pigmented spots in six patients [15].
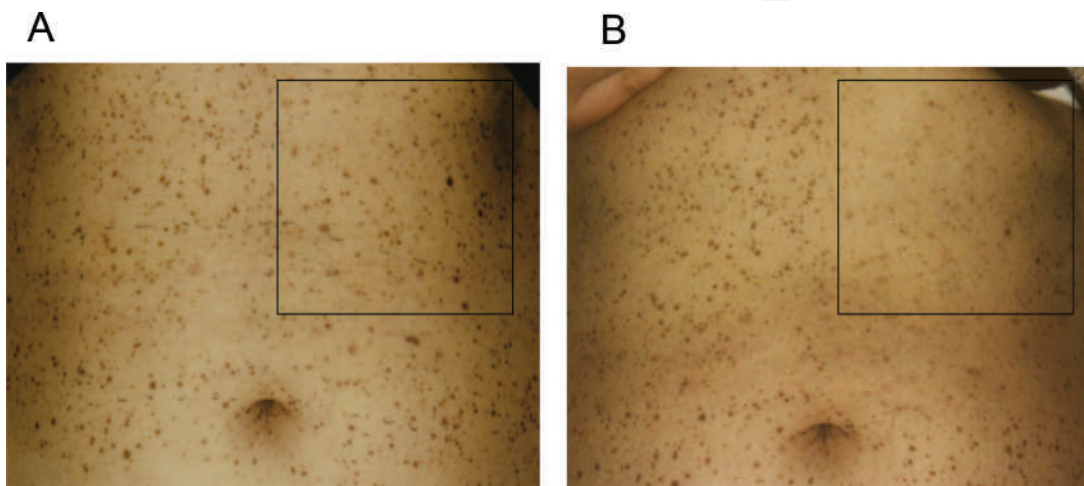

Figure 9. Effects of combining IPL-RF irradiation with topical OCT treatment on multiple pigmented spots on the torso of a 27-year-old female NF1 patient. Six courses of irradiation with a light fluence of 15–25 J/cm^2 and RF energy 15–25 J/cm^2 were carried out over a 4-month period. Irradiation of 70–100 shots/100 cm^2 was performed once every 2 or 4 weeks. Photographs shown were taken before (A) and 6 months after (B) treatment.

With regard to the effects of topical VD3 analogs in combination with laser irradiation on CALMs, we experienced one case of a young male patient who had a conspicuous CALM on his nose. The patient had previously been treated with Q-switched Ruby laser irradiation in a cosmetic clinic for a long time without success before he visited our dermatology clinic at Fukuoka University Hospital. The pigmented macule was first treated only with topical OCT application twice a day for 10 months. After that, Q-switched Ruby laser irradiation (8 J/cm^2) was performed twice every 3 months along with continuing topical OCT application. The CALM showed fair improvement after the initial 10 months of topical OCT application and then virtually disappeared over the course of the next 6 months with combination treatment (**Figure 10**).

Recently, an irradiation procedure known as laser toning has been applied to alleviate facial melasma of women for cosmetic purposes [16, 17]. Given that our previous experience using laser toning with an Nd:YAG laser for melasma brought about fairly good results, we applied this procedure to CALMs and small pigmented freckles on seven NF1 patients in combination with topical tacalcitol application. Side-by-side therapy with either laser toning alone or in

combination with tacalcitol treatment was adopted. A typical case, a 17-year-old female patient with NF1, is shown in **Figure 11**.
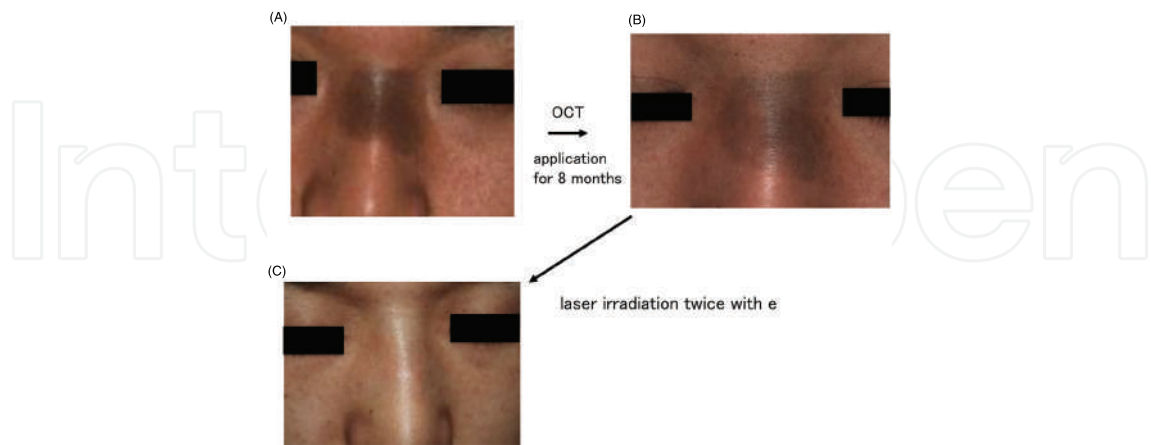

Figure 10. CALM on the nose of a 20-year-old male NF1 patient treated with OCT in combination with Q-switched Ruby laser irradiation. A) Before treatment, (B) after 10 months of topical OCT application twice a day, and (C) after 6 months of Q-switched Ruby laser irradiation twice every 3 months in combination with topical OCT treatment.

Figure 11. Side-by-side treatment of hyperpigmentation and small pigmented spots using Nd:YAG laser toning with or without topical tacalcitol ointment. Pigmented lesions of a 17-year-old female patient were treated seven times with laser toning only (right side) or seven times with laser toning in combination with topical tacalcitol ointment (left side). Pigmentation of her left side was more improved compared with the right side.

Her facial pigmented spots were treated with either laser toning alone (right side) or laser toning plus tacalcitol treatment (left side, **Figure 11**). Hyperpigmentation around her mouth and small pigmented spots around her neck became lighter on the left side compared with those on the right. Since laser toning with an Nd:YAG laser exerts effects mainly on epidermal melanocytes, this treatment was thought to have little beneficial effect on CALMs. However,

side-by-side therapy revealed that a combination of laser toning with topical tacalcitol application caused more lightening in the examined area compared with laser toning treatments alone (**Figure 12**).
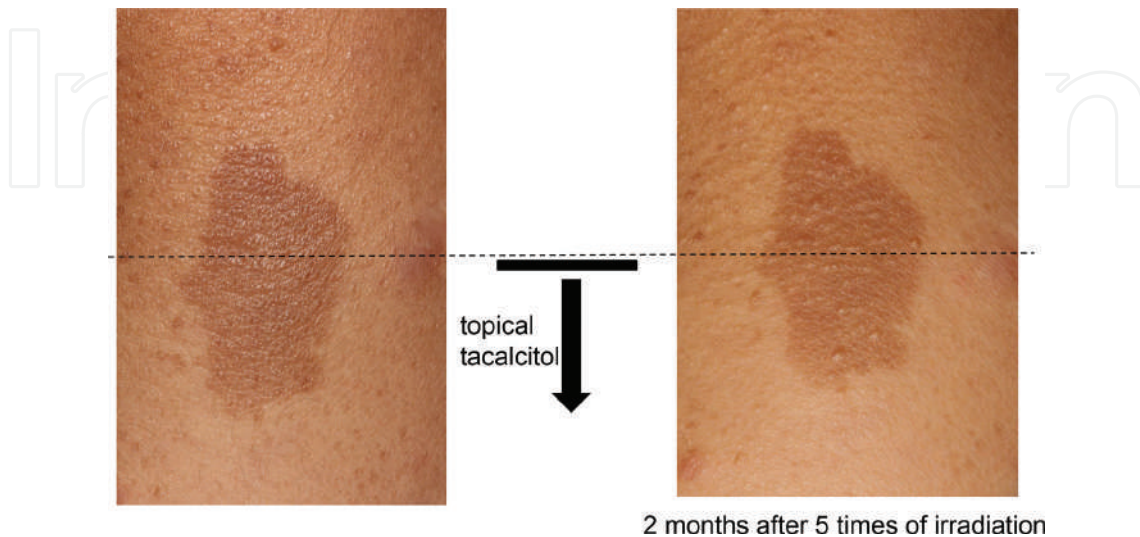
Figure 12. Side-by-side treatment of a CALM using Nd:YAG laser toning with or without topical tacalcitol ointment. A CALM of a 28-year-old male patient was treated five times with laser toning only (upper half) or five times with laser toning and topical tacalcitol ointment (lower half).

4. Discussion

NF1 is one of the major neurocutaneous syndromes. Patients with this disease experience decreased quality of life caused by cutaneous morbidities such as CALMs, skin-fold freckling, and NFs. Moreover, if malignant peripheral nerve sheath tumors should arise from plexiform NFs, it is potentially lethal to patients with NF1. Our understanding of the etiopathogenesis of NF formation in NF1 has progressed with use of mouse models and various cells isolated from NFs. Tumors are comprised of Schwann cells, mast cells, fibroblasts, and perineurial cells; however, Schwann cells are thought to be the primary tumor cell-type as they possess both germline and second-hit mutations in the NF1 gene (NF1$^{-/-}$) [18]. Other components, such as mast cells and fibroblasts with haploinsufficient NF1 gene mutations (NF1$^{+/-}$) are essential to sustain the formation of NFs. In a mouse model, Nf1$^{-/-}$ diploinsufficient Schwann cells rapidly proliferated and secreted KIT ligand at approximately sixfold higher levels compared with wild-type controls [19]. Mast cells haploinsufficient for the NF1 gene infiltrated into NFs in response to KIT ligand and exhibited potency to proliferate. Precise etiopathogenesis of CALMs remains obscure, but it has been suggested that melanocyte density is increased within CALMs [20]. Also, somatic mutation analysis yielded two NF1 hits in melanocytes isolated from CALMs [21]. A one-hit mutation in melanocytes has been thought to cause skin-fold freckling and global hyperpigmentation.

VD3 and its analogs have been found to inhibit in vitro growth of primarily isolated fibroblasts and mast cells, but not Schwann cells. We also have found that growth of commercially available human epidermal melanocytes is efficiently inhibited by VD3 and its analogs. We used these human epidermal melanocytes in our study because it was difficult to obtain informed consent from patients to excise a large enough sample of a CALM to get the required number of melanocytes. Thus, whether the growth inhibition rate of primarily isolated melanocytes (NF1$^{+/-}$) from NF1 patient CALMs is the same as that of the human epidermal melanocytes (NF1$^{+/+}$) remains to be examined.

Given that we observed that the growth of Schwann cells was not affected by VD3 and its analogs, we extended our study further to whether an mTOR inhibitor (rapamycin) or Ras-MEK pathway inhibitor (lovastatin) could inhibit the growth of primary Schwann cells and fibroblasts isolated from NFs. It was found that these agents could inhibit growth of both Schwann cells and fibroblasts isolated from NFs. A combination of VD3 with rapamycin and/or lovastatin did not increase the suppressive effects of either of these drugs on the growth of Schwann cells, but it did cause additive suppression of fibroblast growth. With regard to clinical use of VD3 or its analogs for NF1 patient skin lesions, our in vitro experimental results indicate a combination of rapamycin and/or lovastatin with VD3 should be more effective at suppressing NF growth in vivo. Although the tumorigenic cells in NFs are considered to be NF1$^{-/-}$ Schwann cells, supporting NF1$^{+/-}$ mast cells and/or fibroblasts are considered to be essential for NF formation.

We found definite inhibitory effects of 308-nm UVB irradiation (excimer light) on growth of all cells comprising the NF1 phenotype in vitro. We then studied whether NB-UVB irradiation brought about beneficial effects on skin lesions of patients with NF1. In addition, we measured changes in serum VD3 levels of these patients after long-term whole body NB-UVB irradiation. We observed that at least 6 months of irradiation significantly increased serum VD3 levels, which were accompanied by a lightening of generalized hyperpigmentation of the skin of most patients examined. Hyperpigmentation commonly resulting from UV or sun exposure, caused by induction of endothelin-1, was not detected with NB-UVB irradiation at doses of 0.2–0.5 J/cm^2 once weekly or biweekly, even if NB-UVB irradiation was continued for more than 3 years.

Increases in NF1 patient serum VD3 levels by NB-UVB irradiation are in accord with a previous report suggesting NB-UVB irradiation for patients with either atopic dermatitis or psoriasis causes upregulation of serum 25-hydroxyvitamin D (calcidiol) [22]. Other reports suggest serum calcidiol levels in patients with NF1 are significantly lower than those of control subjects [23], and low levels of calcidiol have a negative correlation with severity of NF formation [24]. Also, reduced bone density and increased incidence of calcidiol deficiency in adults with NF1 have been reported [25]. Therefore, oral supply of VD3 and long-term NB-UVB irradiation could bring about benefits for both skin and internal lesions of NF1 patients; although, care should be taken to identify and minimize any adverse events caused by long-term NB-UVB irradiation.

To date, no evidence supports use of laser therapy for removal of CALMs. However, we experienced the virtual disappearance of a CALM on the nose of a young male patient with

NF1 after two treatments with Q-switched Ruby laser irradiation in combination with continuous topical OCT application during a 16-month period. We also observed multiple small pigmented spots on the torso of a female patient with NF1 virtually disappeared after treatment with IPL-RF in combination with topical OCT application. Our recent studies using laser toning with an Nd:YAG laser in combination with topical tacalcitol application showed fairly good results with regard to multiple facial small pigmented spots. So, we recommend adopting this irradiation with topical VD3 ointment for pigmented spots associated with severe cosmetic problems, especially those on the face, in patients with NF1.

Our clinical investigative results with VD3 have been obtained from a small number of patients with NF1, and no double-blind studies have been performed. Therefore, further studies of a larger scale are needed to clarify to what extent pigmented lesions such as CALMs, small pigmented spots, and skin-fold freckling can be improved by irradiation with laser or IPL-RF in combination with VD3. Additional studies examining suppression of new NF formation with long-term whole body NB-UVB irradiation, which significantly enhances serum VD3 levels in patients with NF1, would also be of great value. It is generally considered that either topical application or internal ingestion of VD3 should exert the same biological mechanism of action. On the one hand, direct topical application of VD3 or its analogs onto skin lesions may be more effective than internal ingestion because of locally higher concentrations of VD3. On the other hand, skin lesions related with NF1, such as CALMs or NFs, are generally scattered across the whole body, ingestion, or injection of VD3 may be more useful for practical therapeutic applications. In conclusion, use of VD3 or its analogs is encouraging for either improving pigmented skin lesions or suppressing new NF formation in patients with NF1.

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