Prolonged Activation of ERK Contributes to the Photorejuvenation Effect in Photodynamic Therapy in Human Dermal Fibroblasts

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Photodynamic therapy (PDT) is known to be effective in the photorejuvenation of photoaged skin. However, the molecular mechanisms of rejuvenation by PDT remain elusive. In this study, we aimed to understand the molecular events occurring during the photorejuvenation after PDT in dermal fibroblasts *in vitro*. First, we found that PDT conditions resulted in an increased fibroblast proliferation and motility *in vitro*. Under this condition, cells had increased intracellular reactive oxygen species (ROS) production. Importantly, PDT induced a prolonged activation of extracellular signal–regulated kinase (ERK) with a corresponding increase in matrix metalloproteinase (MMP)-3 and collagen type Iα messenger RNA and protein. Moreover, inhibition of PDT-induced ERK activation significantly suppressed fibroblast proliferation and expression of MMP-3 and collagen type Iα following PDT. In addition, NAC (an antioxidant) inhibited PDT-induced fibroblast proliferation and ERK activation indicating that prolonged ERK activation and intracellular ROS contribute to the proliferation of fibroblasts and the dermal remodeling process for skin rejuvenation. We also identified increased collagen volume and decreased elastotic materials that are used as markers of photoaging in human skin samples using histochemical studies. Results from this study suggest that intracellular ROS stimulated by PDT in dermal fibroblasts lead to prolonged activation of ERK and, eventually, fibroblast proliferation and activation. Our data thus reveal a molecular mechanism underlying the skin rejuvenation effect of PDT.

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INTRODUCTION

UV irradiation from the sun damages human skin and causes premature skin aging (photoaging). Changes in collagen, the main fibrillar components of the connective tissues and the extracellular proteins of the skin, have been proposed as a main source of the skin wrinkling observed in photodamaged skin (Fisher *et al.*, 1997; Varani *et al.*, 2000). UV irradiation increases the levels of matrix metalloproteinases (MMPs) in human skin *in vivo* (Fisher *et al.*, 1996), and MMPs are responsible for collagen damage in UV-irradiated human skin. MMP is a class of enzymes responsible for the degradation of extracellular matrix proteins such as collagen type I and III (Gu *et al.*, 2011). MMP-1 is the main enzyme that degrades collagen in the skin (Kähäri and Saarialho-Kere, 1997), and once MMP-1 breaks down collagen, further degradation is followed by MMP-3 and other MMPs (Sternlicht and Werb, 2001).

Photodynamic therapy (PDT) is a noninvasive technique used in the treatment of various skin disorders. Clinical studies have reported over years that the PDT has effects on the photorejuvenation of aged skin (Dover *et al.*, 2005; Gold *et al.*, 2006). Despite a few investigations on the subject, this therapy remains controversial for the treatment of skin aging, largely because of the uncertainty of its fundamental cellular and molecular mechanisms. Topical PDT using 5-aminolevulinic acid (ALA) is based on the photosensitization of the diseased tissue by ALA-induced porphyrins and subsequent irradiation with red light. The excitation of the photosensitizer results in the generation of reactive oxygen species (ROS) and ROS then mediate cellular effects such as lipid peroxidation and vascular effects, resulting in direct or indirect cytotoxic effects on the treated cells (Brackett and Gollnick, 2011).

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Abbreviations: ALA, 5-aminolevulinic acid; ERK, extracellular signal–regulated kinase; JNK, c-Jun N-terminal kinase; MAPKs, mitogen-activated protein kinases; MMPs, matrix metalloproteinases; PDT, photodynamic therapy; ROS, reactive oxygen species

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ROS have been increasingly acknowledged to control signal transduction through the activation of mitogen-activated protein kinases (MAPKs) (Pan *et al.*, 2009). Three distinct, although related, families of MAPKs exist: extracellular signal–regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 kinase. There are extensive cross-talks among the 3 families of MAPKs. Among them, the ERK pathway primarily mediates cellular responses to growth factors (Xia *et al.*, 1995), whereas the JNK and p38 pathways primarily mediate cellular responses to cytokines and physical stress (Xia *et al.*, 1995; Verheij *et al.*, 1996). One earlier study has demonstrated that ERK activity is reduced in aged human skin *in vivo* versus young skin, whereas JNK activity is increased (Shin *et al.*, 2005). Several studies have indicated that MAPK pathways have an important role in regulating cell growth (Xia *et al.*, 1995) and procollagen synthesis (Davis *et al.*, 1996; Chen and Davis, 1999). MAPK has been also known to have an important role in the signaling pathways that regulate MMP-1 gene expression (Lam *et al.*, 2005). Among the MAPK subgroups, both ERK1/2 and p38 MAPK have been known to regulate MMP gene expression (Brennan *et al.*, 2003).

We thus hypothesized that enhanced but low level of ROS at inner depths of the human skin after the PDT may activate cellular functions of dermal fibroblasts through the MAPK and ERK pathways, and that activation of ERK signaling may have a stimulatory effect on the proliferation and activation of dermal fibroblasts. The purpose of this study is to investigate these possible molecular mechanisms of the skin rejuvenation by PDT in dermal fibroblasts.

RESULTS

Treatment of low-level PDT enhanced the proliferation of fibroblasts

Topical PDT is based on the photosensitization of diseased tissue by ALA-induced porphyrins and subsequent irradiation (Gold *et al.*, 2006). We first tested the cytotoxicity of ALA and light irradiation in primary human dermal fibroblasts. ALA treatment alone up to 1 mM did not induce any morphological signs of cell cytotoxicity, and cell viability was not substantially decreased as measured by the 3-(4,5-dimethyl-2-thiazol-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Figure 1a). We then measured irradiation-induced cell cytotoxicity in the fibroblasts. After treatment with different irradiation dose and at different distances, cell viability was measured by the MTT assay. As shown in Figure 1b, these conditions did not induce cytotoxic effect in fibroblasts. When the cells were treated with ALA alone, light alone, and PDT, respectively, cells did not show any cytotoxic effects as evidenced by morphology (Figure 1c and Supplementary Figure S1 online). Fibroblasts are known to have a crucial role in mediating skin rejuvenation, ranging from their proliferation to remodeling of extracellular matrix such as type I collagen (Kim *et al.*, 2009). To investigate the effect of PDT on the proliferation of normal human fibroblasts, a combination of various PDT conditions (ALA concentration (0, 0.05, 0.1, and 0.5 mM) and red-light level (0, 3, and 10 J cm^{-2})) were evaluated to determine their effects on the fibroblast density by cell counting and morphology. The highest rate of fibroblast proliferation was observed at the ALA concentration of 0.1 mM, incubation time of 30 minutes, and 3 J cm^{-2} of irradiation among the various PDT conditions (Supplementary Figure S2 online). These PDT conditions were of lower intensity compared with the *in vitro* PDT conditions reported previously (Karrer *et al.*, 2003). Proliferation of fibroblasts was examined by the MTT assay and proliferating cell nuclear antigen (PCNA) immunofluorescence staining (Figure 1d and e). As shown in Figure 1d, PDT increased the proliferation of fibroblasts as compared with the untreated group (control vs. PDT, $P<0.05$). However, treatment with ALA alone did not markedly change the proliferation of fibroblasts compared with the control group, and light alone had a minimal effect. PDT also increased the expression of PCNA compared with the control group, with no significant influence of light alone or ALA alone on the expression of PCNA as compared with the untreated control (Figure 1e). In addition, a scratch wound assay detected an increased motility of fibroblasts in the PDT group (Figure 1f). Our data suggest that PDT may increase both the proliferation and the motility of fibroblasts with regard to its effects during the photorejuvenation of aged skin.

Treatment of low-level PDT induced prolonged ERK activation in fibroblasts

Several studies indicate that MAPK signaling pathways have an important role in regulating cell proliferation (Stork and Schmitt, 2002). To explore the underlying mechanism of the proliferation effect of PDT, we examined the changes of MAPK activation upon PDT conditions in fibroblasts. PDT resulted in a rapid and prolonged activation of phospho-ERK up to 8 hours. However, although light alone increased short-term ERK activation, ERK activation was not prolonged as with full PDT, and ALA itself had little or no effect (Figure 2a and Supplementary Figure S3 online). We also investigated the effect of PDT on other MAPKs including JNK and p38. As shown in Figure 2b, PDT had no effect on the activity of JNK and p38 MAPKs. However, all of these kinases activated in fibroblasts were treated with tumor necrosis factor-α (Figure 2c). In addition, nuclear staining of phosphor ERK was substantially increased as identified by immunohistochemical and immunofluorescence analyses (Figure 2d). These results clearly indicate that the low-level PDT is a potent and specific inducer of the ERK pathway but not JNK and p38.

PDT-induced ERK activation contributes to the proliferation of fibroblasts

To further prove the role of ERK activation in fibroblast proliferation, we next examined the effect of the ERK inhibitor PD98059 on fibroblasts proliferation after PDT. To do this, cells were pretreated with PD98059 for 30 minutes, followed by treatment with PDT for 4 hours. PD98059 efficiently inhibited phosphorylation of ERK upon PDT (Figure 3a). Increased PCNA expression after PDT was significantly suppressed by PD98059 (Figure 3b). As shown in Figure 3c, increased cell motility by PDT was also markedly decreased in

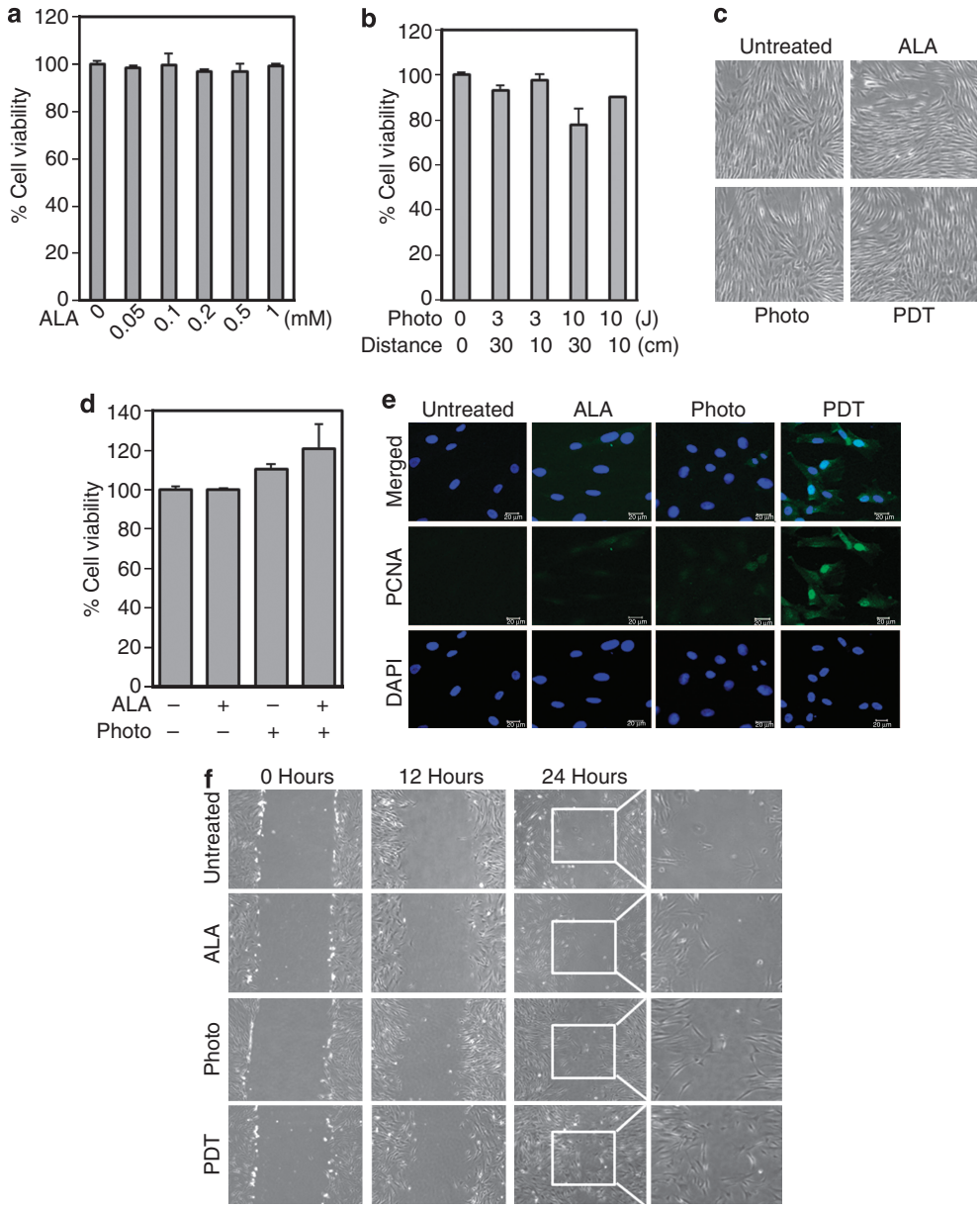

Figure 1. Low-level photodynamic therapy (PDT) enhanced proliferation of human dermal fibroblasts. (**a**) 5-Aminolevulinic acid (ALA) itself had no cytototoxic effect on primary dermal fibroblasts. Cells were treated with different concentrations of ALA (up to 1 mM) for 12 hours and cell cytotoxicity was measured by the MTT assay. Results are mean ± SEM. (**b**) The effect of irradiation dose of the light and distances from the light source in fibroblasts. Cells were treated with different irradiation dose of the light and distances from the light source for 12 hours and cell cytotoxicity was measured by the MTT assay. Results are mean ± SEM. (**c**) PDT induced no morphological change. Fibroblasts were treated with ALA at the concentration of 0.1 mM, irradiation at the energy of 3 J cm^{-2}, and PDT at the ALA concentration of 0.1 mM, incubation time of 30 minutes, and 3 J cm^{-2}, respectively. After 12 hours of each treatment, representative images were taken by a phase-contrast microscope. (**d**, **e**) PDT induced fibroblast proliferation. Cells were treated with PDT (0.1 mM ALA and 3 J cm^{-2} light) and the proliferation of fibroblasts was measured by MTT assays (**d**). Results are mean ± SEM. Cell proliferation also analyzed by proliferating cell nuclear antigen (PCNA) immunofluorescence staining and representative images were taken by confocal microscope (**e**). (**f**) PDT enhanced fibroblast motility. Fibroblasts were evaluated to determine their effects on the motility using a wound-healing assay. All data shown are representative of three independent experiments. Bar = 20 μm. DAPI, 4′6-diamidino-2-phenylindole; PCNA, proliferating cell nuclear antigen.

the presence PD98059 as measured by the scratch wound assay. Taken together, our data indicated that prolonged ERK activation by low-level PDT induced the proliferation and motility of the fibroblasts that may be the main mechanism of skin photorejuvenation.

PDT-induced generation of ROS attributes to the prolonged ERK activation and the enhanced proliferation of fibroblasts

It has been suggested that ERK activation may be a general signaling defense against oxidative stress, and activation of ERK can confer a survival advantage to cells (Xia *et al.*, 1995;

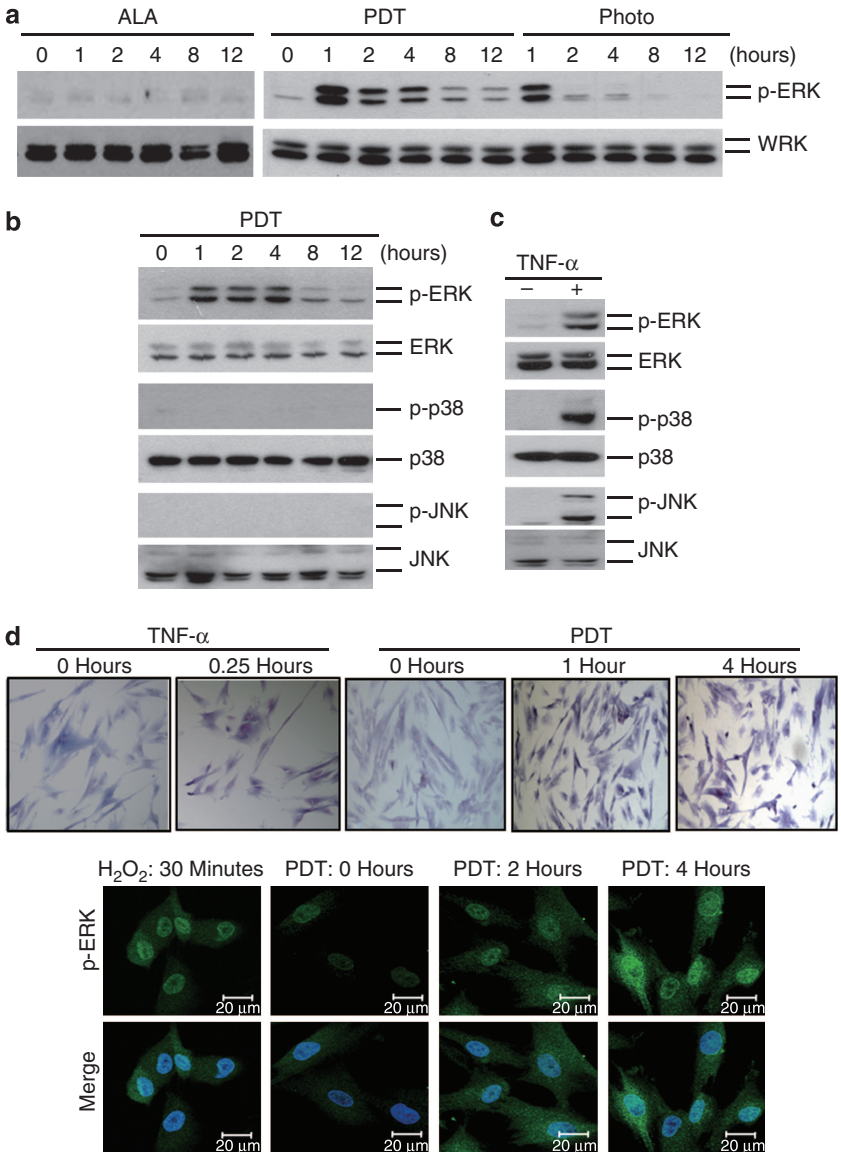

Figure 2. Photodynamic therapy (PDT) induced prolonged extracellular signal–regulated kinase (ERK) activation in human dermal fibroblasts. (**a**, **b**) PDT induced prolonged ERK activation. Cells were treated with 5-aminolevulinic acid (ALA) only, light only, and ALA and light (PDT condition: 0.1 mM ALA and 3 J cm^{-2}) for indicated time points and cell lysates were analyzed by western blot. (**c**) Tumor necrosis factor-α (TNF-α) induced activation of mitogen-activated protein kinases (MAPK) in dermal fibroblasts. Cells were treated with TNF-α (30 ng ml^{-1}) for 30 minutes and cell lysates were analyzed by western blotting as a control of MAPK activation. (**d**) Nuclear localization of ERK is induced by PDT in fibroblasts. Cells were treated with indicated conditions and immunohistochemical and immunofluorescence analyses were done by phosphor ERK (p-ERK) antibody. Bar = 20 μm. JNK, c-Jun N-terminal kinase.

Verheij *et al.*, 1996). As our data indicate that PDT-induced prolonged ERK activation contributes to cell proliferation and cell motility, it is possible that PDT-generated ROS stimulates MAPK activation. We measured ROS generation by FACS using 2′,7′-dichlorfluorescein-diacetate staining for low-level PDT conditions at different time points. As shown in Figure 4a (left panel), fibroblasts treated with low-level PDT showed increased intracellular ROS production in a time-dependent manner, but amounts of ROS were much lower than those in cells treated with H_2O_2 for 30 minutes. PDT-induced ROS generation was also visualized with confocal microscopy (Figure 4a right panel and Figure 4b). As shown in Figure 4b, an evident increase in 2′,7′-dichlorfluorescein-diacetate fluorescent intensity was observed in the PDT group, whereas ALA alone had little effect on fluorescent intensity.

It has been suggested that low-level ROS could contribute to fibroblast proliferation and that NADPH oxidase (Nox) activity also could control the dermal fibroblast proliferation. In order to examine the potential involvement of ROS in the proliferative response to low-level PDT, we investigated the effect of antioxidants, NAC, on fibroblast proliferation following PDT. PDT-induced ERK activation was abolished by NAC pretreatment (Figure 4c) and increased PCNA expression after PDT was significantly suppressed by NAC indicating that PDT-induced ROS generation contributes to ERK activation–mediated fibroblast proliferation (Figure 4d).

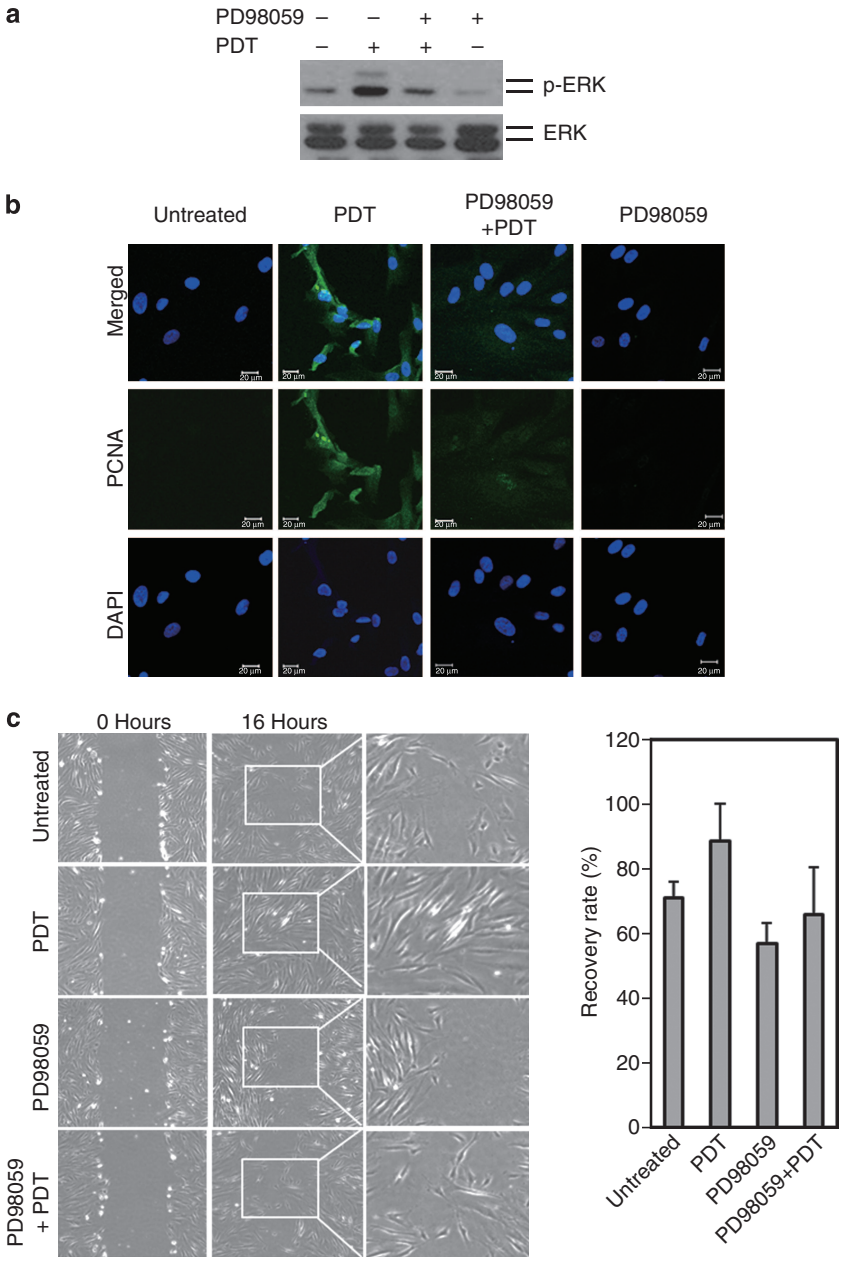

Figure 3. Photodynamic therapy (PDT)-induced prolonged extracellular signal–regulated kinase (ERK) activation contributes fibroblast proliferation and motility. (**a**) ERK inhibitor PD98059 effectively blocked ERK activation. Fibroblasts were pretreated with PD98059 (20 μM) for 30 minutes, followed by treatment with PDT for 4 hours. Cell lysates were analyzed by western blot. (**b**) Inhibition of ERK activation by PD98059 abolished PDT-induced cell proliferation. Fibroblasts were pretreated with PD98059 for 30 minutes, followed by treatment with PDT 24 hours, and cell proliferation was analyzed by proliferating cell nuclear antigen (PCNA) immunofluorescence staining; representative images were taken by confocal microscope. (**c**) PDT-induced prolonged ERK activation enhanced fibroblast motility. Fibroblasts were evaluated to determine effects on motility using a wound-healing assay. All data shown are representative of three independent experiments. Scratched cells were pretreated with PD98059 (20 μM) for 30 minutes, followed by treatment with PDT for 16 hours, and representative images were taken by phase-contrast microscope. Under microscope, distance of wound was measured as five different points to quantify recovery rate. Recovery rates are expressed as mean ± SEM from duplicate determinations of three independent experiments for each case. Bar = 20 μm. DAPI, 4′6-diamidino-2-phenylindole; p-ERK, phosphor ERK; PCNA, proliferating cell nuclear antigen.

Moreover, the addition of NAC and BHA after a low-level PDT significantly suppressed fibroblast motility (Figure 4e). Further, we examined whether Nox activity is involved in fibroblast proliferation and motility upon PDT. To test this, we checked Nox1 expression levels upon PDT. As shown in Supplementary Figure S4a online, PDT did not significantly alter Nox1 expression level, but, similar to antioxidant treatment, the NADPH oxidase inhibitor DPI inhibited ERK activation and cell motility upon PDT (Supplementary Figure S4b and c online). As PDT is thought to directly initiate ROS

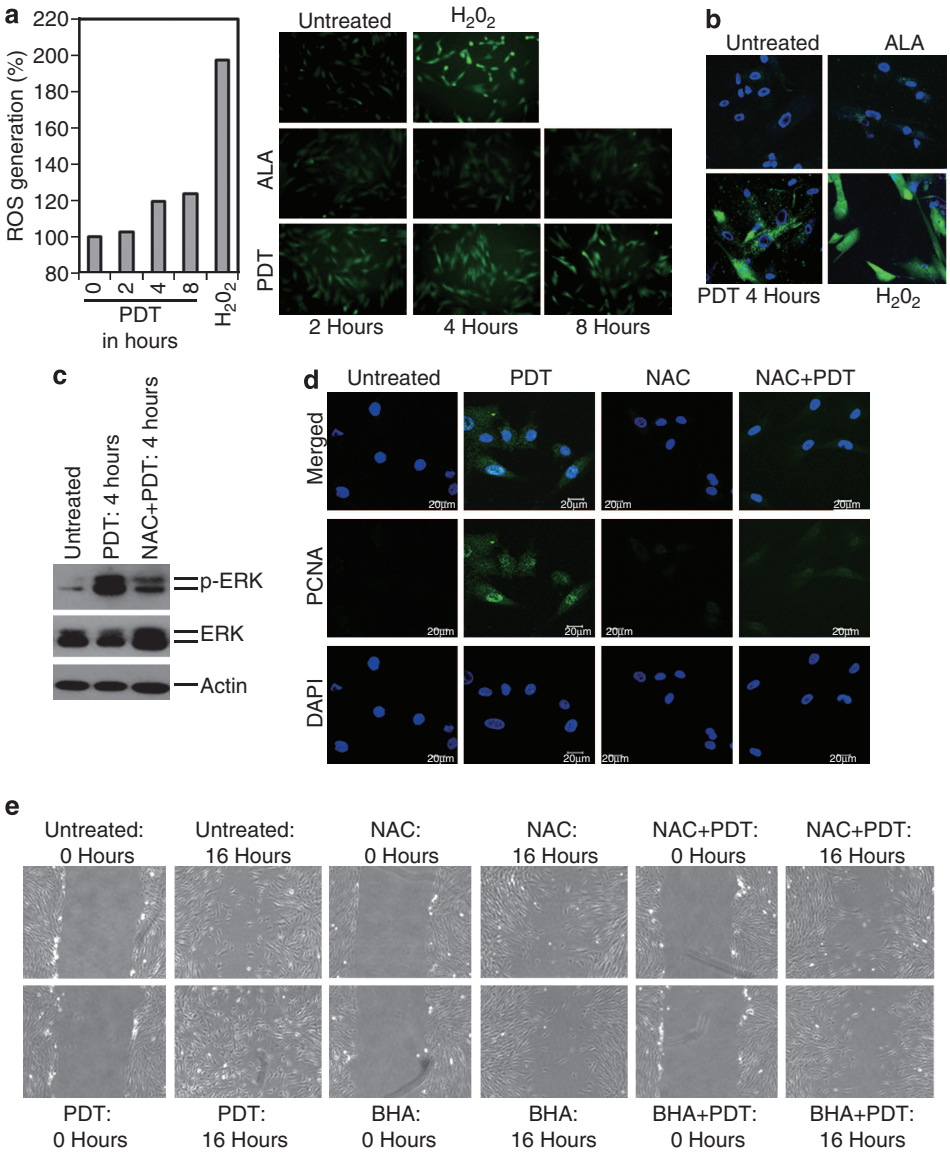

Figure 4. Intracellular reactive oxygen species (ROS) by photodynamic therapy (PDT) contributes fibroblast proliferation through prolonged extracellular signal–regulated kinase (ERK) activation. (**a**, **b**) PDT induced intracellular ROS generation in fibroblasts. Cells were treated with PDT for different time points and ROS generation was measured by FACS using the DCFH-DA. As a control, cells were treated with H_2O_2 (0.5 mM) for 30 minutes and 5-aminolevulinic acid (ALA) alone also evaluated for ROS generation. ROS was visualized with fluorescence microscopy (**a**) and confocal microscopy (**b**). (**c**) ROS by PDT induced ERK activation in fibroblasts. Cells were pretreated with NAC (1 mM) for 30 minutes, followed by treatment with PDT for 4 hours and cell lysates were analyzed by western blotting. (**d**) Inhibition of ROS generation by antioxidant abolished PDT-induced fibroblast proliferation. Cells were pretreated with NAC (1 mM) for 30 minutes, followed by treatment with PDT for 24 hours and cell proliferation analyzed by proliferating cell nuclear antigen (PCNA) immunofluorescence staining and representative images were taken by confocal microscope. (**e**) Inhibition of ROS generation by antioxidant abolished PDT-induced fibroblast motility. Scratched cells were pretreated with NAC (1 mM) for 30 minutes, followed by treatment with PDT for 16 hours and evaluated to determine their effects on the motility using a wound-healing assay. Representative images were taken by phase-contrast microscope. All data shown are representative of three independent experiments. Bar = 20 μm. DAPI, 4′6-diamidino-2-phenylindole; NAC; p-ERK, phosphor ERK; PCNA, proliferating cell nuclear antigen.

formation, it is unclear as to how Nox1 activity contributes to ERK activity and PDT-induced fibroblast proliferation and motility, but it is possible that Nox-mediated ROS generation could contribute through amplification of the initial ROS, as has been shown for small amounts of hydrogen peroxide (Li *et al.*, 2001). Nevertheless, our results suggest that intracellular ROS generated by PDT attributes to the prolonged ERK activation and enhanced proliferation of fibroblasts.

PDT modulated MMP activity and collagen synthesis

It has also been suggested that collagen damage occurs during natural skin aging, as it does during photoaging, due to elevated MMP expression, and that total MMP levels are elevated in human sun-protected aged skin *in vivo* versus comparable younger skin (Chung *et al.*, 2001). Among these indicated MMPs, we performed reverse transcriptase–PCR, real-time PCR and western blot to evaluate the changes in

MMP-1, MMP-2, MMP-3, and MMP-12 following PDT. We found no significant changes in MMP-1, MMP-2, and MMP-12 messenger RNA (mRNA) up to 24 hours after PDT (Figure 5a). In addition, PDT had no influence on protein levels of MMP-1 and MMP-12 up to 72 hours following PDT (Figure 5b). However, MMP-3 mRNA levels changed significantly, with a maximal induction (about 3.7-fold as compared with the control) at 12 hours after PDT (Figure 5c top panel), and protein levels of MMP-3 were increased at 24 hours after PDT (Figure 5c bottom panel). More significantly, a substantial and time-dependent induction of collagen type Iα mRNA levels (up to 2.1-fold after 24 hours) and protein was observed after PDT (Figure 5d). It is possible that an increase in MMP-3 may therefore promote the degradation and removal of old, damaged collagen fibers, while the cell is initiating formation of new ones to replace them (Saito *et al.*, 1998). Alternatively MMP-3 expression may contribute to other types of remodeling within the tissue or to fibroblast motility.

As our data indicate that PDT induces generation of intracellular ROS, which modulates prolonged ERK activation, PDT could therefore regulate MMP-3 and collagen type Iα expression through ERK signaling. To investigate the

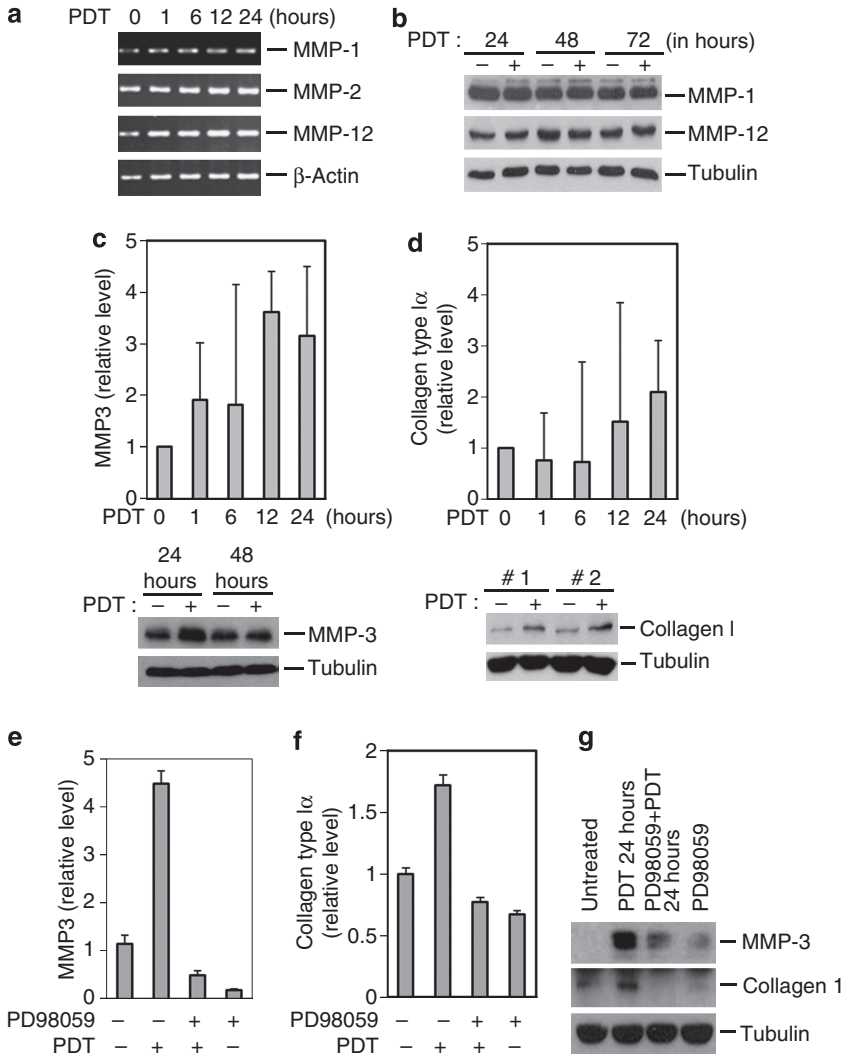

Figure 5. Matrix metalloproteinase-3 (MMP-3) and collagen type Iα messenger RNA (mRNA) and protein levels after the photodynamic therapy (PDT) were upregulated in fibroblasts. (**a**) No influence of PDT on the levels of MMP-1, 2, and 12 mRNA. Cells were treated with PDT for indicated time points and RNA was isolated and analyzed by reverse transcriptase–PCR (RT-PCR). (**b**) No effect of PDT on the MMP-1 and 12 protein levels. Cells were treated with PDT for indicated time points and cell lysates were analyzed by western blot. (**c**) PDT-induced upregulation of mRNA and protein levels of MMP-3. Cells were treated with PDT for indicated time points, mRNA level was analyzed by real-time PCR, and protein level was analyzed by western blotting. (**d**) Upregulation of collagen type Iα mRNA and protein levels by PDT. Cells were subjected to PDT for the indicated time points, mRNA level was analyzed by real-time PCR, and protein level was analyzed by western blotting. (**e**, **f**) Inhibition of extracellular signal–regulated kinase (ERK) activation by PD98059 suppressed upregulation of MMP-3 and collagen type Iα mRNA after the PDT. Cells were pretreated with PD98059 (20 μM) for 30 minutes, followed by treatment with PDT (MMP-3 for 24 hours (**e**) and collagen type Iα for 48 hours (**f**)), and mRNA levels were analyzed by real-time PCR. (**g**) PDT-induced ERK activation caused changes in connective tissue matrix components. Fibroblasts were pretreated with PD98059 (20 μM) for 30 minutes, followed by treatment with PDT for 24 hours. Cell lysates were analyzed by western blotting.

connection between ERK activation and the upregulation of MMP-3 and collagen type Iα, cells were pretreated with PD98059 for 30 minutes, followed by treatment with PDT. As shown in Figure 5e and f, ERK inhibition efficiently inhibited MMP-3 and collagen type Iα mRNA expression. Similarly, expression of PD98059 suppressed PDT-induced upregulation of MMP-3 and collagen type Iα proteins in fibroblasts (Figure 5g). These results suggest that prolonged ERK activation may directly contribute to increased MMP-3 and collagen type Iα expression in fibroblasts indicating that PDT may activate cellular functions of dermal fibroblasts through MAPK pathways.

PDT resulted in histological changes suggesting recovery of photodamaged human skin

Furthermore, we investigated whether PDT induced histological changes during the rejuvenation of human skin. Five patients with clinical features of photoaged skin, such as dryness, wrinkling, telangiectasia, irregular pigmentation, and AK lesions on the face were recruited into the study. All patients received two sessions of PDT every four weeks. Skin biopsy was performed before and 1 month after the last session of PDT. Clinically, all patients showed improvement in both aged skin and AK lesions following two sessions of PDT (Figure 6a). In hematoxylin and eosin-stained sections, dysplastic keratinocytes in AK lesions were cleared after PDT, and there was a tendency of decrease within these elastotic masses compared with the pretreatment specimens (Figure 6b). We also identified a well-organized collagen band beneath the basal membrane which was not observed before treatment (Figure 6c). After PDT, a marked decrease in elastotic materials was noted, which was a pathognomonic sign for photoaging in human skin. These data suggest that PDT is effective treatment in skin rejuvenation in clinical applications.

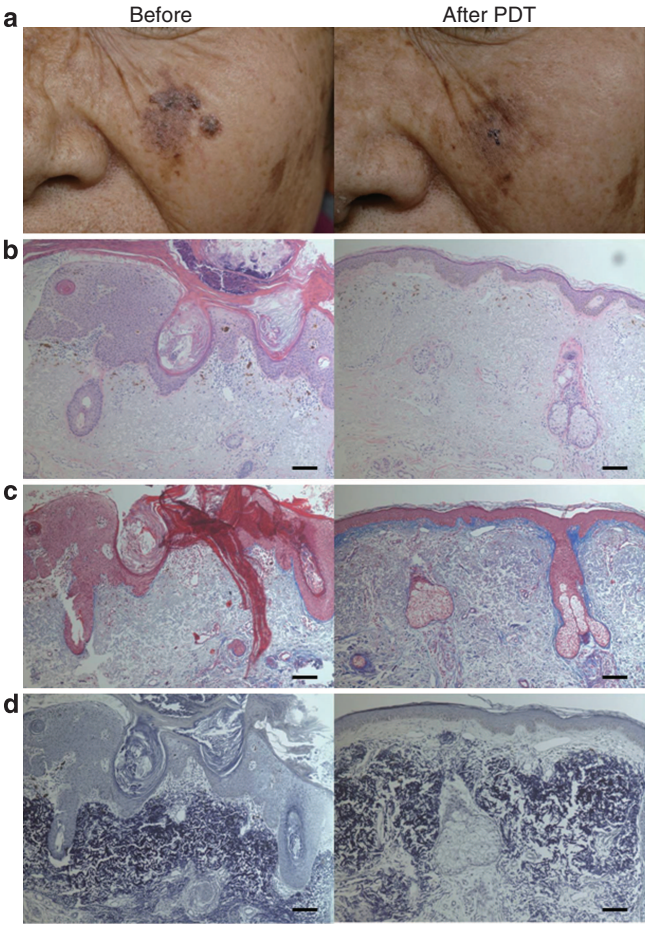

Figure 6. Photodynamic therapy (PDT) resulted in histological changes indicating restoration of photoaged human skin. Skin biopsy specimens before and 1 month after the PDT were examined with hematoxylin-eosin, Masson's trichrome collagen stain, and Verhoeff's-van Gieson elastic stain and were evaluated by the parameters associated with photoaging. Specimens on the left are before PDT; specimens on the right are after PDT. (**a**, **b**) After ALA-PDT, actinic keratosis lesion and skin texture were improved clinically and histologically (H&E stain; original magnification ×200). (**c**) Masson-trichrome stained specimens demonstrated upregulated collagen fibers in upper dermis (original magnification ×200). (**d**) PDT reduced the accumulation of dystrophic elastotic materials in the dermis (Verhoeff's stain; original magnification ×200). Bar = 100 μm. ALA, 5-aminolevulinic acid.

DISCUSSION

In this study, we found that fibroblast proliferation was induced by low-level PDT, which generates intracellular ROS to induce prolonged ERK activation. In clinical applications, fibroblast proliferation would not be the therapeutic objective but rather modulation of collagen synthesis. However, as PDT-induced fibroblast proliferation can serve as a starting point for future investigations, it was important to identify the dosimetry parameters. In addition, *in vitro*-enhanced proliferation of dermal fibroblasts may reflect the proliferation phase of skin rejuvenation *in vivo*.

ROS can cause decreased cellular proliferation and increased cytotoxicity. However, several previous reports showed the paradoxical functions of ROS as intracellular second messengers and signaling molecules in various intracellular processes (Burdon and Rice-Evans, 1989; Burdon *et al.*, 1990). Several experiments have shown that relatively low and controlled concentrations of ROS can stimulate cellular proliferation (Ding *et al.*, 2001; Kim *et al.*, 2001). In the *in vivo* state, when at inner depths of the tissue, low amounts of ROS may form after the PDT; these low amounts of ROS might stimulate fibroblasts and collagen metabolism, resulting in an improvement of skin texture. We found that intracellular ROS generated by low-level PDT contributed to the enhanced proliferation of fibroblasts. Fibroblast proliferation was inhibited by addition of antioxidants indicating growth-stimulatory properties of low amounts of ROS generated after a low-level PDT. Several studies also suggested that tumor necrosis factor-α induces superoxide formation in skin fibroblast and the generation of superoxide radicals is involved in causing biological changes in connective tissue matrix components (Sundaresan *et al.*, 1996, Taniguchi *et al.*, 1996). Nox enzymes are one of the main cellular sources of ROS and they could contribute to cell proliferation. In the present study, it remains unclear whether NADPH oxidases, such as Nox1, have a role in PDT-induced

cell proliferation and biological changes in connective tissue matrix components. However, the NADPH oxidases inhibitor DPI abolished PDT-induced ERK activation, as well as cell motility indicating possible involvement of Nox, likely Nox1, which is expressed in dermal fibroblasts.

ROS have been acknowledged as controlling signal transduction through the activation of MAPK to transcription factors (Maziere *et al.*, 2003). A previous study reported that the balance between the ERK activity and the JNK and p38 kinase activities shifted in favor of reduced cell growth and increased stress response in aged human skin (Shin *et al.*, 2005). We showed that the ERK pathway remained activated after PDT, like ROS. In addition, we demonstrated PDT-induced ERK activation was suppressed by antioxidant pretreatment. Therefore, it is very likely that prolonged production of ROS after PDT induces prolonged activation of the ERK pathways, which may contribute to skin rejuvenation *in vivo*.

Human fibroblasts have the capacity to produce several MMPs including MMP-1 (interstitial collagenase), MMP-2 (72 kDa gelatinase), and MMP-3 (stromelysin-1). Previously, we reported that the intensity of MMP-1, MMP-3, and MMP-12 immunostaining decreased following treatment with PDT in human photoaged skin (Park *et al.*, 2010). However, in present study, we found that MMP-3 protein levels increased after PDT, even though PDT did not affect the regulation of MMP-1, 2, and 12. This conflicts with the findings of our *in vivo* study. The results regarding increased levels of MMP-3 might be explained in several ways. First, the *in vivo* study might differ from *in vitro* study because of *in vivo* considerations. In addition, our previous study assessed the histological alterations one month after PDT. The time point of the evaluation must be considered as a significant factor in analysis. A recent *in vivo* PDT study in human skin showed an acute increase in the levels of MMP-1 mRNA, which returned to baseline levels within 24 hours (Orringer *et al.*, 2008). We speculate that when MMP-3 is formed immediately after PDT, it destroys old collagen fibers and may allow for the new collagen synthesis. Therefore, PDT-induced upregulation of MMP-3 may reflect the initial phase of PDT-induced rejuvenation process in human skin.

Since our study assessed the effects of PDT only on fibroblasts, we could not evaluate cellular interaction between fibroblasts and other cutaneous cells including keratinocytes, melanocytes, dermal dendritic cells, monocytes, and perivascular lymphocytes. For example, various cytokines and chemokines from other skin cells such as keratinocytes might be indirectly affected on dermal fibroblasts. Accordingly, the *in vitro* findings of the present study could only reflect the acute effects of PDT on human dermal fibroblasts that might occur *in vivo*. The results of study suggest a role of prolonged ERK activation and intracellular ROS production in the molecular mechanism of photodynamic skin rejuvenation. PDT on photoaged skin produces ROS at the deeper skin layers. In sequence, intracellular ROS induce ERK activation in dermal fibroblasts. ERK activation promotes fibroblast proliferation and increased secretion of the MMP-3. Increasing MMP-3 expression may accelerate old extracellular matrix destruction. Proliferated fibroblasts may also induce production of new collagen construction. Although a few previous studies evaluated the photorejuvenation effects of PDT, the precise molecular mechanism has remained unknown. The findings in our study represent early evidence clarifying these cellular mechanisms, or at least a partial explanation for the skin rejuvenation effects of PDT *in vivo*.

MATERIALS AND METHODS

Reagents

Recombinant tumor necrosis factor-α was purchased from R&D Systems (Minneapolis, MN). Anti-phopho-JNK, anti-phospho-ERK, anti-phospho-p38, anti-ERK, and anti-p38 antibodies were purchased from Cell Signaling (Beverly, MA), MA. Anti-JNK antibody was purchased from Invitrogen (Carlsbad, CA). Anti-MMP-1, 3, and 12 antibodies were purchased from Epitomics (Burlingame, CA). Anti-tubulin and anti-collagen type I antibodies were purchased from Abcam (Cambridge, MA). NAC, BHA, DPI, and ALA were purchased from Sigma. PD 98059 was purchased from Calbiochem.

Cell culture

Primary human normal dermal fibroblasts were previously described (Palmetshofer *et al.*, 1995). Fibroblasts were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. Fibroblasts were subcultured by trypsinization and used between the 5th and 10th passages.

PDT procedure

Cells were plated in 60-mm dish. After the growth medium was removed, serum-free medium containing various concentrations of ALA (0.05–1 mM) was added, and cells were allowed to take up ALA for 30 minutes. The medium containing ALA was removed; the cells were rinsed and then submerged with phosphate-buffered saline. Irradiation with various light intensities (3–10 J cm^{-2}) was performed immediately afterward. Irradiation of the cells within monolayer culture was performed using an incoherent light source (Omnilux revive, 633 nm, Phototherapeutics, Montgomeryville, PA).

Cell proliferation and viability assay

Cell viability was determined using the tetrazolium dye colorimetric test (MTT assay). The MTT absorbance was then read at 570 nm. Representative images were taken by a phase-contrast microscope. The expression of PCNA in the fibroblasts was also detected by immunofluorescence staining using a polyclonal antibody against PCNA (Leica Microsystems, Wetzlar, Germany), and representative images were taken by confocal microscope.

Cell motility assay

The migration capabilities of primary human dermal fibroblasts were assessed using a scratch wound assay, which measures the expansion of cell population on surfaces. The cells were seeded into 60 mm dishes and cultured to reach confluence. Thereafter, a linear wound was generated in the monolayer with a sterile 100 μl plastic tip. After the PDT, cells were incubated and photographed at different time points. The representative images from the scratched areas under each condition were photographed to estimate the relative motility. Recovery rate was quantified by ratio between the end point of wound distance and the initial wound distance (each condition group were measured five different points).

Western blot analysis

Upon PDT, cells were lysed in M2 buffer (20 mM Tris at pH 7, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM DTT, 0.5 mM PMSF, 20 mM β-glycerol phosphate, 1 mM sodium vanadate, and 1 μg ml^{-1} leupeptin). Equal amounts of cell extracts were resolved by 12% SDS-PAGE and analyzed by western blotting and visualized by enhanced chemiluminescence (Amersham).

Immunohistochemical staining

Fibroblasts were incubated on chamber slides and fixed by cold acetone for 10 min. Immunohistochemical staining was performed using avidin–biotin–peroxidase complex. For the immunofluorescence staining, primary antibody to phospho-ERK (Cell signaling) was reacted for overnight at 4 °C, and FITC-conjugated secondary antibody (goat anti-mouse IgG, 1:500 dilution, Molecular Probes) were reacted for 1 hour at room temperature. Representative images were taken by confocal microscope.

Measurement of intracellular ROS

To detect intracellular ROS, we incubated cells with 10 μM H2 DCFDA for 30 minutes before the end of the indicated treatments and the increase in fluorescence was measured by FACS and the cells were examined under a confocal microscope (Carl Zeiss LSM710, Jena, Germany).

Reverse transcriptase–PCR

RNA was extracted with the RNeasy Kit (Qiagen, Hilden, Germany). One microgram of total RNA from each sample was used as a template for cDNA synthesis with a reverse transcriptase kit (Invitrogen). An equal amount of cDNA product was used in the PCR performed using the Taq DNA polymerase (Takara, Otsu, Japan). PCR amplification was performed using the primers the listed in Supplementary Table S1 online. The final PCR products were resolved in 1.5% agarose gel and stained with ethidium bromide.

Real-time PCR

Specific messages were amplified and detected by real-time PCR performed using the SYBR Green method (Applied Biosystems, Foster City, CA) using specific sets of forward and reverse primers (Supplementary Table S2 online) synthesized at Bioneer (Daejeon, Korea). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was used as a control to normalize for the amount of RNA present in various test samples. The relative value of specific mRNAs in each sample was expressed as a reciprocal ratio of the Ct values of each message to those of the corresponding glyceraldehyde-3-phosphate dehydrogenase.

Histochemistry

After obtaining an informed consent, 4 mm punch skin biopsy specimens were taken from 5 patients (before and after two sessions of PDT). For histological analyses in human tissues, skin biopsy specimens were fixed in 10% formalin, embedded in paraffin, and cut into 5 μm-thick sections. Sections were stained with hematoxylin-eosin, Masson's trichrome collagen stain, and Verhoeff's-van Gieson elastin stain.

Statistics

Independent experiments were performed at least in triplicates. Statistical significance was evaluated in paired analyses using the Mann–Whitney *U* test (nonparametric), depending on the data distribution. Data values are expressed as the mean ± SEM. Statistical significance was defined as a $P<0.05$.

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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