PDT-activated calpain was responsible for the sequential activation of caspase cascade and apoptosis-inducing factor (AIF) by damaging mitochondria, which resulted in caspase-dependent and caspase-independent apoptotic cell death. Both the caspase cascade and the activation of AIF translocated to the nucleus and, synergistically with the caspase-dependent pathway, led to apoptotic cell death. Both the caspase cascade and the activation of AIF following PDT were mediated by TG2 activation. In addition, PDT-activated calpain was responsible for the sequential events of Bax translocation, the collapse of \( \Delta \Psi_m \), caspase-3 activation, and AIF translocation, all of which were provoked by TG2 activation. Together, these results demonstrate that PDT with a chlorin-based photosensitizer targets TG2 by activating calpain-induced Bax translocation, which induces apoptotic cell death through both caspase-dependent and AIF-mediated pathways. Moreover, these results indicate that TG2 may be a possible therapeutic target for PDT treatment of cancer.

Transglutaminase 2 (TG2), the most ubiquitous member of the transglutaminase family, catalyzes post-translational protein cross-linking with the formation of \( \varepsilon-(\gamma\text{-}L-glutamyl)lysine \) or \( \gamma\text{-}L-glutamyl \)polyamine bonds. This transamidating activity of TG2 regulates a variety of biological responses, including cell death, cell adhesion and proliferation, cytoskeletal reorganization, and cell migration and invasion. TG2 is implicated in neurodegenerative diseases, celiac disease, ischemic stroke and cardiovascular diseases, cataractogenesis, diabetes, and cancer. Understanding the function of TG2 as a therapeutic target will contribute to improved cancer treatments using PDT.
TG2/Calpain/Bax-dependent Apoptotic Cell Death

caspase-dependent and caspase-independent apoptosis are not clearly understood.

Photodynamic therapy (PDT) can induce not only routine caspase-dependent apoptosis but also caspase-independent apoptosis, depending on the photosensitizer and the cell context. PDT generally causes apoptosis by activating a caspase cascade, a result of the release of cytochrome c through the mitochondrial permeability transition (13–15), which is controlled by Bcl-2 family members. On the other hand, by damaging mitochondria, PDT induces the release of AIF into the cytosol and its subsequent nuclear translocation, leading to apoptosis in a caspase-independent manner (16). Similar results were observed in cells loaded with the phthalocyanine derivative 2,3,9,10,16,17,23,24-octakis(3-aminopropoxy)phthalocyaninato zinc II following PDT. PDT damages lysosomes as a primary target and, in turn, induces Bid activation, mitochondrial dysfunction, and nuclear translocation of AIF, resulting in caspase-independent apoptosis (17). Furthermore, studies have indicated that the caspase-dependent pathway and the AIF-mediated pathway are simultaneously involved in the apoptotic death of cancer cells following PDT (18, 19). However, the mechanisms underlying the simultaneous induction of the two pathways and the mechanism by which AIF is regulated during PDT remain to be elucidated.

We recently presented a possible role of TG2 in apoptotic cell death following PDT with a chlorin-based photosensitizer localizing in the endoplasmic reticulum, mitochondria, and lysosomes. The activity of TG2 may mediate the apoptotic death of cancer cells through a mechanism coupled with increases in intracellular reactive oxygen species (ROS) and Ca\(^{2+}\) levels (20). However, the signaling mechanism by which TG2 regulates apoptotic cell death during PDT is not clearly understood. In this study, we elucidated the apoptotic signaling pathways mediated by TG2 activation in human cancer cells during PDT with a photosensitizer. TG2 promoted apoptotic cell death in response to PDT through a signaling cascade involving the calpain/Bax signaling pathway due to increased transamidating activity, but not due to increased TG2 expression levels. The resulting activation of calpain was sufficient to induce the collapse of the mitochondrial membrane potential (ΔΨ\(_{m}\)) through the translocation of Bax to mitochondria, which ultimately induced both caspase-dependent and caspase-independent death pathways.

**EXPERIMENTAL PROCEDURES**

**Materials and Cell Culture**—5-(Biotinamido)pentylamine and FITC-conjugated streptavidin were purchased from Pierce. Benzoxycarbonyl-VAD-fluoromethyl ketone (Z-VAD-fmk) was obtained from Alexis Biochemicals (San Diego, CA). Propidium iodide, cystamine, and Trolox were purchased from Sigma, and BAPTA-AM was purchased from Calbiochem. The antibodies used were obtained as indicated: anti-TG2 (NeoMarkers, Fremont, CA), anti-AIF and anti-caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-cytochrome c and anti-Bax (Pharmingen). Anti-caspase-9 monoclonal antibody and anti-poly(ADP-ribose) polymerase polyclonal antibody were from Cell Signaling Technology (Beverly, MA). Human gastric adenocarcinoma AGS and human bladder carcinoma J82 cells (American Type Culture Collection) were maintained at 37 °C in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (culture medium) in a 5% CO\(_2\) atmosphere.

**PDT with Chlorin-based Photosensitizer**—The photosensitizer DH-II-24 (2,3-dimethylaminooxytocarbonyl-13\(^{\beta}\)-demethoxy carbonyl-phosphoribide) was kindly provided by Professor Chang-Hee Lee (Kangwon National University). PDT was performed using an on-stage system as reported previously (20). Briefly, cells were incubated for 12 h with 1 μg/ml DH-II-24 and irradiated at 1.45 milliwatt/cm\(^2\) for 60 s (87 mJ/cm\(^2\)) in darkness using a built-in 100-watt halogen lamp with an LP630 filter (Fiber Optic Korea Co., Ltd.). DH-II-24 alone (without irradiation) was used as a negative control.

**Transfection with siRNAs**—Cells were transfected with siRNAs following the procedures of Yi et al. (21). Briefly, TG2-specific siRNA duplexes (5’-AAGGCAGAGAUCG-GAAC-3’) targeting the coding sequence of human TG2 mRNA, AIF-specific siRNAs (ON-TARGET plus SMART pool), and control siRNA (ON-TARGET plus non-targeting pool) were obtained from Dharmacon (Lafayette, CO). Cells were transfected with siRNAs using siLentFect lipid reagent (Bio-Rad) according to the manufacturer’s instructions. Twenty hours after transfection, the media were replaced with fresh culture medium, and the transfected cells were further incubated for 24 h prior to experimentation.

**In Situ Transglutaminase Activity Assay**—In situ transglutaminase activity was determined by a confocal microscopic assay following the procedures of Yoo et al. (20). Briefly, cells were incubated with 1 mM 5-(biotinamido)pentylamine for 1 h at 37 °C, and biotinylated proteins were probed with FITC-conjugated streptavidin. Single-cell fluorescence intensities were determined by confocal microscopy (Olympus Fluoview FV300) in ~30 cells from three separate experiments. Relative TG2 activity was determined by comparing the fluorescence intensities of irradiated cells with those of non-irradiated control cells (fold differences).

**Cell Viability Assay**—Cell viability assays were performed as described previously (20). Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays and is expressed as the percentage of that of non-irradiated control cells.

**Western Blot Analysis**—To obtain cell lysates, cells were incubated for 30 min in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 0.1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Cells were scraped and centrifuged at 20,000 × g for 10 min at 4 °C. Cytosolic and mitochondrial extracts were prepared using the Thermo Scientific Mitochondria Isolation Kit for Cultured Cells (Pierce) according to the manufacturer’s instructions.

Cell lysates and fractionated extracts were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dried milk in 20 mM Tris (pH 7.6), 138 mM NaCl, and 0.1% Tween 20 for 1 h at room temperature. Proteins were probed with antibodies against the target proteins. Protein bands were visualized by exposure to x-ray film and a chemiluminescent substrate (Pierce).
Determination of $\Delta \Psi_m$ was monitored by confocal microscopy with the potential-sensitive JC-1 cationic dye (Molecular Probes, Eugene, OR), which exhibits potential-dependent accumulation in mitochondria. Mitochondrial depolarization (loss of $\Delta \Psi_m$) is indicated by an increase in the ratio of the fluorescence intensity of the monomeric form (green) to that of the J-aggregates (red). To monitor $\Delta \Psi_m$, DH-II-24-loaded cells were irradiated with the built-in halogen lamp and incubated with 2 $\mu$g/ml JC-1 dye for 30 min at 37 °C. Fluorescence intensities of the cells were determined at the single-cell level using FluoView FV300 software with a confocal microscope. Data are expressed as relative fluorescence intensities and the ratio of the fluorescence intensity of monomers to that of J-aggregates.

**Immunostaining and Confocal Microscopy**—Apoptotic cells were identified by confocal microscopy with the BD Pharmlin-gen FITC Caspase-3, Active Form, mAb Apoptosis Kit I according to the manufacturer’s instructions. For quantification of apoptosis, at least 300 cells were selected from three independent experiments, and the percentages of apoptotic cells were calculated.

To analyze AIF translocation, cells were fixed with 3.7% formaldehyde in PBS for 30 min, permeabilized with 0.2% Triton X-100 in PBS for 30 min, and incubated with blocking solution (2% BSA in TBS containing 0.1% Triton X-100) for 30 min. Cells were incubated with anti-AIF antibody in blocking solution, probed with FITC-conjugated anti-mouse IgG, and further incubated with 10 $\mu$g/ml propidium iodide. Stained samples were observed by confocal microscopy. Approximately 20 cells were randomly selected from each of three independent experiments, and the fluorescence intensities of the cells were measured by processing only their FITC images at the single-cell level using FluoView FV300 software. AIF translocation was expressed as the ratio of the AIF fluorescence intensity of the nucleus to that of the whole cell.

**Calpain Activity Assay**—The in vitro activity of calpain (Promega, Madison, WI) was measured according to the manufacturer’s instructions. Briefly, cell extracts were obtained using lysis buffer containing 50 mM HEPES (pH 7.5), 1% Triton X-100, 150 mM NaCl, and 1 mM EDTA and incubated for 15 min with equal volumes of Calpain-Glo™.
reagent, including succinyl-LLVY-aminoluciferin (a succinyl proluminescent calpain substrate), and 2 mM CaCl₂ (final concentration of 1 mM). The luminescence of each sample was recorded in relative light units by a microplate luminescence reader (Molecular Devices, Sunnyvale, CA). Calpain activity was determined by comparing the luminescence intensities of irradiated cells with those of non-irradiated control cells (fold differences).

Statistical Analysis—Data are expressed as means ± S.D. Statistical significance was analyzed using one-way analysis of variance (Origin 6.1 software, OriginLab Corp., Northampton, MA). p values of <0.05 were considered statistically significant.

RESULTS

Role of TG2 in Stimulation of Caspase-dependent Apoptotic Pathway by DH-II-24-mediated PDT—Because PDT-induced apoptotic death of AGS cells may be prevented by inhibiting caspase activation (20), we asked whether DH-II-24-mediated PDT induces caspase-dependent apoptotic cell death. The contribution of well characterized apoptotic factors to the cytoxic response to PDT was assessed by Western blotting (Fig. 1A). PDT resulted in a release of cytochrome c from the mitochondria to the cytosol in a time-dependent manner. This event was accompanied by procaspase-9 cleavage into active caspase-9. In addition, the activation of caspase-3 and the
cleaved product of poly(ADP-ribose) polymerase were detected in a similar time-dependent manner. However, TG2 expression did not significantly change during this period (Fig. 1A). Using a FITC-conjugated active caspase-3 monoclonal antibody apoptosis kit, we further demonstrated that PDT stimulated caspase-3 in a time-dependent manner, with maximal stimulation at 12 h (Fig. 1B). These results suggest that DH-II-24-mediated PDT stimulates caspase-dependent apoptotic cell death in AGS cells.

The potential role of TG2 in the PDT-induced activation of caspase-3 was examined using various inhibitors and TG2 siRNA. Because intracellular ROS and Ca²⁺ are required for the activation of TG2 by DH-II-24-mediated PDT in AGS cells (20), we used a ROS scavenger, Trolox, and a Ca²⁺ chelator, BAPTA-AM. The PDT activation of caspase-3 was completely abrogated by Trolox and was significantly inhibited by BAPTA-AM (Fig. 1C). Consistent with a previous report (22), caspase-3 activation was prevented by cystamine. As expected, the broad-spectrum caspase inhibitor Z-VAD-fmk completely inhibited caspase-3 activation. However, the inhibitor had no effect on the activation of TG2 by PDT (data not shown). In addition, the activation of caspase-3 by PDT was suppressed by TG2 siRNA in a dose-dependent manner, but transfection with TG2 siRNA did not affect the basal caspase-3 activity (Fig. 1D). Control siRNA had also no effect on the activation of caspase-3 by PDT. Transfection with TG2 siRNA suppressed the expression of TG2 in a dose-dependent manner (Fig. 1E). Therefore, TG2 is required for caspase-dependent apoptotic cell death induced by DH-II-24-mediated PDT.

Role of TG2 in AIF-mediated Apoptotic Cell Death Induced by DH-II-24-mediated PDT—Although apoptotic cell death caused by DH-II-24-mediated PDT is caspase-dependent, it may also involve other apoptotic factors, such as AIF, because cell shrinkage was observed in a fraction of cells treated with Z-VAD-fmk (data not shown). To verify this possibility, we investigated whether PDT could induce AIF release from the mitochondria into the cytosol. PDT resulted in AIF release into the cytosol in a time-dependent manner, but AIF expression levels were unaffected during this period (Fig. 2A). Nuclear translocation of AIF was confirmed by confocal microscopy (Fig. 2B).

We examined the possible role of TG2 in PDT-induced nuclear translocation of AIF by analyzing the effects of various inhibitors on the ratio of the AIF fluorescence intensity in the nucleus to that in the whole cell (Fig. 2C). Nuclear translocation of AIF was inhibited by Trolox, BAPTA-AM, and cystamine, but not by Z-VAD-fmk. Additionally, AIF nuclear translocation was prevented by TG2 siRNA in a dose-dependent manner (Fig. 2D). Thus, TG2 is involved in the nuclear translocation of AIF by DH-II-24-mediated PDT.

To determine whether PDT induces AIF-mediated apoptotic cell death, AGS cells were transfected with AIF siRNA. Transfection with AIF siRNA strongly suppressed the expression of AIF in a dose-dependent manner (Fig. 3A). However, AIF siRNA had no inhibitory effects on TG2 activation by PDT, whereas Trolox, BAPTA-AM, and cystamine consistently inhibited TG2 activation (Fig. 3B). We studied the effects of AIF siRNA on apoptotic cell death induced by PDT. Transfection

FIGURE 3. AIF-mediated apoptotic cell death induced by DH-II-24-mediated PDT. AGS cells were transfected with human AIF siRNA or control siRNA (Ctrl-siRNA; 100 nM); loaded with 1 μg/ml DH-II-24 for 12 h; and irradiated (IR) for 60 s in the presence of 100 μM Z-VAD-fmk (Z-VAD), 5 mM Trolox, 5 μM BAPTA-AM (BAPTA), or 100 μM cystamine. A, siRNA concentration-dependent inhibition of AIF expression. B, effects of various inhibitors and AIF siRNA (100 nM) on PDT-induced activation of TG2. In situ TG2 activity was determined 2 h after PDT. TGase, transglutaminase. C, dose-dependent inhibitory effects of AIF siRNA on decreased cell viability induced by PDT. D, effects of Trolox, Z-VAD-fmk, AIF siRNA (100 nM), and a combination of Z-VAD-fmk and AIF siRNA on cell death induced by PDT. Data represent means ± S.D. from three independent experiments. **, p < 0.01.
with AIF siRNA significantly inhibited the decrease in cell viability caused by PDT in a dose-dependent manner (Fig. 3C). Interestingly, the combination of Z-VAD-fmk and AIF siRNA enhanced the inhibition of decreased cell viability induced by PDT (Fig. 3D). Thus, DH-II-24-mediated PDT induces apoptotic cell death through both caspase-dependent and AIF-dependent pathways involving TG2 activation.

**Loss of ΔΨₘ Induced by DH-II-24-mediated PDT**—DH-II-24 accumulates in the mitochondria of AGS cells (20), and PDT induced the release of cytochrome c (Fig. 1A) and AIF (Fig. 2A) from the mitochondria into the cytosol, suggesting that PDT may damage mitochondria. Thus, we investigated whether a loss of ΔΨₘ is associated with PDT-induced apoptotic cell death. The loss of ΔΨₘ increased with time following PDT; the amount of green fluorescent monomers increased, whereas J-aggregates with red fluorescence mostly disappeared in a time-dependent manner (Fig. 4A).

We evaluated a possible role for TG2 in the PDT-induced loss of ΔΨₘ by calculating the ratio of monomers to J-aggregates. The loss of ΔΨₘ was inhibited by Trolox, BAPTA-AM, and cystamine, but not by Z-VAD-fmk (Fig. 4B), indicating a possible role of TG2. TG2 siRNA also prevented the loss of ΔΨₘ in a dose-dependent manner (Fig. 4C). These results demonstrate that DH-II-24-mediated PDT induces apoptotic cell death through a mitochondria-mediated intrinsic pathway that is regulated by TG2.

**Calpain Is Involved in Apoptotic Cell Death Induced by DH-II-24-mediated PDT**—Calpain is involved in AIF release from mitochondria (23, 24), and PDT induced AIF-dependent apoptotic death of AGS cells (Fig. 3). Thus, we examined whether calpain is activated following DH-II-24-mediated PDT. PDT activated calpain in a time-dependent manner, with maximal activation at 3 h (Fig. 5A). As expected, a selective calpain inhibitor, PD150606, inhibited the activation of calpain, but not TG2, by PDT (Fig. 5B). The activation of calpain by PDT was significantly prevented by Trolox, BAPTA-AM, and TG2 siRNA, but not by Z-VAD-fmk (Fig. 5C), indicating that TG2 functions as an upstream signal of calpain.

To determine whether calpain is involved in TG2-dependent apoptotic responses to PDT, we analyzed the effects of PD150606 on the activation of caspase-3 and the translocation of AIF into the nucleus following PDT. Treatment with PD150606 effectively abrogated both caspase-3 activation and AIF translocation in a dose-dependent manner (Fig. 5D). Therefore, calpain is involved in the TG2-mediated death mechanism induced by DH-II-24-mediated PDT. The role of calpain was further supported by data indicating that PD150606 significantly inhibited the loss of ΔΨₘ (Fig. 5E).
Additionally, the decrease in cell viability induced by PDT was sufficiently blocked in a dose-dependent manner by PD150606 (Fig. 5F). Thus, calpain participates in TG2-dependent apoptotic cell death through caspase- and AIF-dependent pathways in response to DH-II-24-mediated PDT.

**DH-II-24-mediated PDT Induces Translocation of Bax**—We asked whether Bax is involved in apoptotic cell death induced by DH-II-24-mediated PDT. PDT induced the translocation of Bax from the cytosol to mitochondria (Fig. 6A). Bax translocation was significantly blocked by Trolox and TG2 siRNA. In addition, the translocation of Bax was inhibited by the calpain inhibitor PD150606. These results demonstrate that TG2 and calpain are involved in PDT-induced translocation of Bax to the mitochondria.

To confirm the role of TG2 in Bax translocation, we expressed Bax-EGFP and Cox8-DsRed in AGS cells and analyzed the effect of TG2 siRNA on the PDT-induced translocation of Bax by confocal microscopy. Cox8-DsRed was used as a mitochondrial marker (25). Bax-EGFP was co-localized with Cox8-DsRed following PDT, whereas its translocation was inhibited by TG2 siRNA (Fig. 6B). Considering the role of TG2 in calpain activation and AIF translocation following PDT (Figs. 2 and 5), these results demonstrate that DH-II-24-mediated PDT induces Bax translocation from the cytosol to mitochondria via activation of TG2 and calpain. Bax translocation to mitochondria results in mitochondrial permeability transition, which leads to a disruption in ΔΨₘ and subsequently induces a mitochondria-dependent death pathway that includes caspases.
A

|          | Control | IR | Ctrl-siRNA+IR | TG2-siRNA+IR | Trolox+IR | PD+IR |
|----------|---------|----|--------------|--------------|-----------|-------|
| Bax      |         |    |              |              |           |       |
| cytosol  |         |    |              |              |           |       |
| mitochondrial | | |            |              |           |       |
| whole lysates |   | |              |              |           |       |
| β-actin  |         |    |              |              |           |       |

B

Bax-EGFP  Cox8-DsRed  Merged

Control

IR

Ctrl-siRNA +IR

TG2-siRNA +IR

Graphs showing fluorescence intensity (FI) vs. distance (μm) for Bax and Cox8.
and/or AIF (11, 26–29). Therefore, DH-II-24-mediated PDT induces apoptotic cell death through a TG2/calpain/Bax signaling pathway that involves caspase- and AIF-dependent mechanisms.

**Apoptotic Cell Death Mediated by TG2-dependent Mechanism in Human Bladder Carcinoma Cells**—To further explore a broad relevance of the preceding results, we investigated the apoptotic signaling pathway induced by DH-II-24-mediated PDT in human bladder carcinoma J82 cells. Initially, we examined the effect of PDT on cell viability. As shown in Fig. 7A, PDT decreased the viability of J82 cells in a post-irradiation time-dependent manner, and both DH-II-24 alone and light alone had no effect on cell viability (inset).

Next, we investigated the effect of various inhibitors and TG2 siRNA on the activation of TG2 by PDT. Inset, suppression of TG2 expression using siRNA. TGase, transglutaminase. C, effects of TG2 siRNA and PD150606 on the activation of calpain by PDT. D, inhibitory effects of TG2 siRNA and PD150606 on the activation of caspase-3 (open bars) and translocation of AIF (closed bars) by PDT. E, inhibitory effects of PD150606 and TG2 and AIF siRNAs on decreased cell viability induced by PDT. Inset, suppression of AIF expression using siRNA. F, effects of Trolox, Z-VAD-fmk, AIF siRNA, and a combination of Z-VAD-fmk and AIF siRNA on cell death induced by PDT. Data represent means ± S.D. from three independent experiments. **, p < 0.01.

**FIGURE 6. Bax translocation to mitochondria induced by DH-II-24-mediated PDT.** A, effects of 200 nm TG2 siRNA, 5 mM Trolox, and 200 μM PD150606 (PD) on the translocation of Bax by PDT. IR, irradiated; Ctrl-siRNA, control siRNA. B, confocal microscopic observation of Bax translocation. A549 cells were transfected with Bax-EGFP and Cox8-DsRed (a mitochondrial marker) constructs, treated with PDT, and observed by confocal microscopy. Scale bar = 15 μm. Fluorescence intensity (FI) profiles of Bax-EGFP (green lines) and Cox8-DsRed (red lines) were obtained by scanning the merged images along the arrows.
TG2 is a multifunctional protein implicated in a number of cellular events, including apoptosis and cell adhesion, survival, migration, and invasion (1–3). TG2 overexpression and activation have been linked to induction of apoptosis (3, 11, 12, 33). Recently, we reported a possible role of TG2 in the PDT-induced apoptotic death of cancer cells (20). However, the detailed mechanism of this apoptotic cell death has not been clearly elucidated. This study clearly demonstrated the essential role of TG2 in apoptotic cell death mechanisms induced by DH-II-24-mediated PDT in human cancer cells. PDT induced apoptotic death of cancer cells through the activation of both caspase-dependent and caspase-independent pathways and required TG2 activation. This process used a TG2-dependent mechanism that involved calpain-mediated translocation of Bax to mitochondria, which resulted in a loss of ΔΨm and the release of both cytochrome c and AIF (Fig. 8).

PDT induces intracellular ROS production and elevates intracellular Ca2+ in various cell types. These messengers stimulate several cytotoxic factors (13, 15). We showed previously that intracellular ROS are required for TG2 activation in fibroblasts (21, 34) and also demonstrated a regulation mechanism of TG2 by intracellular Ca2+ in AGS cells (35). Consistent with these findings, we demonstrated that TG2 activation by DH-II-24-mediated PDT was mediated by intracellular ROS and Ca2+. The ROS scavenger Trolox completely inhibited TG2 activation by PDT, and partial TG2 inhibition by the Ca2+ chelator BAPTA-AM was also observed (20). Furthermore, apoptotic cell death induced by PDT was inhibited by Trolox and partially inhibited by BAPTA-AM (20). Thus, intracellular ROS function as major mediators in PDT-induced intracellular signaling that leads to TG2 activation and apoptotic cell death (Fig. 8).

What is a possible mechanism by which intracellular ROS activate the Ca2+-dependent enzyme TG2? First, direct activation of TG2 was investigated by measuring in vitro TG2 activity in the presence of exogenous H2O2. However, in vitro TG2 activity was not affected by exogenous H2O2 (data not shown), indicating that ROS have no direct effect on TG2 activity in an extracellular environment. Another possible mechanism is that intracellular ROS activate TG2 by regulating other factors, including intracellular Ca2+ levels. Intracellular ROS mediate the elevation of intracellular Ca2+ levels in fibroblasts and skeletal muscle (36, 37). Oxidative stress can activate TG2 by increasing intracellular Ca2+ concentrations and by releasing TGFB2 (38). We observed that Trolox partially suppressed PDT-induced increases in intracellular Ca2+ levels, whereas...
BAPTA-AM had no inhibitory effect on the production of ROS following PDT (20). Moreover, incubation with BAPTA-AM partially inhibited apoptotic cell death induced by PDT (20), indicating that intracellular Ca^{2+} participates, in part, in the cellular responses to PDT. Thus, intracellular ROS are likely to activate TG2 by regulating the levels of intracellular Ca^{2+} and many other factors. It remains necessary to elucidate the exact mechanism of TG2 activation by intracellular ROS during PDT.

In conclusion, we have demonstrated that, following DH-II-24-mediated PDT, TG2 plays a key role in the apoptotic death of cancer cells through a signaling cascade that includes calpain activation and Bax translocation, resulting in mitochondrial dysfunction. The mitochondrial dysfunction stimulates both caspase-dependent and AIF-mediated pathways. These findings contribute to the understanding of the complex cell death pathways induced by PDT and to the improvement of PDT treatment of cancers.

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