Short Communication

Release of Cytochrome c, Bax Migration, Bid Cleavage, and Activation of Caspases 2, 3, 6, 7, 8, and 9 during Endothelial Cell Apoptosis

David J. Granville,^{*††} Janet R. Shaw,^{††} Simon Leong,[‡] Christopher M. Carthy,^{††} Philippe Margaron,[‡] David W. Hunt,^{††} and Bruce M. McManus^{*†}

From the Vancouver Vascular Biology Research Centre-St. Paul's Hospital, Vancouver, British Columbia, Canada; the Department of Pathology and Laboratory Medicine,[‡] University of British Columbia, Vancouver, British Columbia, Canada; and QLT PhotoTherapeutics Inc.,[‡] Vancouver, British Columbia, Canada*

Although the executioner phase of apoptosis has been well defined in many cell types, the subcellular events leading to apoptosis in endothelial cells remain undefined. In the current study, apoptosis was induced in primary human umbilical venous endothelial cells by the photosensitizer verteporfin and light. Release of mitochondrial cytochrome c into the cytosol was detectable immediately and accumulated over 2 hours after treatment while cytosolic levels of the proapoptotic Bcl-2 family member, Bax, decreased reciprocally over the same time period. Cleavage of another proapoptotic Bcl-2 family member, Bid, was observed by 2 hours after treatment. Although Bid cleavage has been shown to occur as an upstream event responsible for inducing cytochrome c release, we demonstrate that Bid cleavage can also occur after cytochrome c release. Activation of caspases 2, 3, 6, 7, 8, and 9 occurred following the release of cytochrome c, and cleavage of downstream substrates was observed. In summary, endothelial cell death involves the cellular redistribution of Bax and cytochrome c, followed by the activation of multiple caspases which manifest the apoptotic phenotype. (*Am J Pathol* 1999, 155:1021–1025)

Endothelial cell (EC) apoptosis appears to be a prominent feature in the pathogenesis of atherosclerosis and transplant vascular disease.^{1,2} Although the cascade of events that occur in many tumor cell lines and immune cells in response to a multitude of different proapoptotic

stimuli has been well described in recent years, many of these events have not been demonstrated for ECs. Caspases (cysteine-specific proteinases) are believed to be the primary effector molecules of apoptosis.^{3,4} Although the status of caspases 1 and caspase 3 in EC death has been examined,^{5–7} the status of other caspase family members is currently unknown. Recently, oxidized low density lipoprotein (OxLDL) was shown to induce the release of cytochrome c from mitochondria during EC apoptosis.⁸ In other cell types, cytosolic cytochrome c, along with apoptotic protease activating factor-1 (Apaf-1) and caspase 9, form a complex termed the "apoptosome" which facilitates caspase 9 activation which then activates downstream caspases such as caspase 3.⁹

A diverse range of stimuli can induce EC apoptosis. In the current study, Photodynamic therapy (PDT) was used as a method to induce apoptosis in human umbilical venous endothelial cells (HUVECs). PDT, using the photosensitizer verteporfin, catalyzes the formation of reactive oxygen intermediates and has been used as a tool to rapidly induce apoptosis in a variety of cell types following light irradiation.^{9–14} PDT is a clinically approved modality for the treatment of various types of cancer. One of the primary targets of PDT during tumor eradication is the tumor neovasculature.¹⁵ PDT is also being investigated for the treatment of atherosclerosis, restenosis, and age-related macular degeneration. However, the effects of PDT on EC apoptosis are poorly understood. In the present report, several novel aspects of EC apoptosis are described, including the cellular redistribution of cyto-

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Address reprint requests to Bruce McManus, Department of Pathology and Laboratory Medicine, Cardiovascular Research Laboratory, University of British Columbia-St. Paul's Hospital, 1081 Burrard Street, Vancouver, BC, Canada V6Z 1Y6. E-mail: mcmanus@unixg.ubc.ca.

chrome c and Bax followed by the activation of multiple caspases resulting in the downstream cleavage of Bid.

Materials and Methods

Reagents

Lipid-formulated verteporfin was provided by QLT PhotoTherapeutics Inc. (Vancouver, BC). Antibodies were obtained from the following sources: caspases 1, 6, and 8 (Upstate Biotechnology Inc., Lake Placid, NY); Bid, caspase 3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA); caspase 7 (Transduction Laboratories, Mississauga, ON); cytochrome c, Bax, caspases 2, 4, 9, and 10 (Pharmingen, Mississauga, ON); poly(ADP-ribose) polymerase (PARP) (Biomol Research Laboratories, Plymouth Meeting, PA).

Cell Culture

HUVECs (Clonetics, San Diego, CA) were maintained in endothelial basal medium (EBM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), hydrocortisone (1 μ g/ml), bovine brain extract (12 μ g/ml), gentamicin (50 μ g/ml), amphotericin B (50 ng/ml), and epidermal growth factor (10 ng/ml) (Clonetics).

Photoactivation of Verteporfin

HUVECs (5×10^6) were incubated for 60 minutes in the dark at 37°C with or without verteporfin (100 ng/ml) in EBM supplemented with 2% FBS. For caspase inhibition studies, 50 μ mol/L Z-Val-Ala-Asp-fluoromethylketone (ZVAD-fmk) (Bachem, Torrance, CA) was added to cells for the final 30 minutes of the verteporfin incubation period before light activation. After drug incubation, cells were exposed to fluorescent red light (620–700 nm) delivered at a rate of 5.6 mW/cm² to give a total dose of 2 J/cm². Cells were then maintained in Petri dishes at 37°C until further analysis.

Immunoblot Analysis

Whole and cytosolic (S-100) cell extracts were prepared as previously described.¹³ Protein concentrations were measured using the Pierce BCA protein assay (Pierce, Rockford, IL). Detergent soluble proteins (30 μ g) were separated by SDS-PAGE in 10 to 12% acrylamide gels under reducing conditions followed by Western blotting as described.¹³ After transfer of protein from the gel to the membrane, gels were stained with 20 ml of Gel-Code Blue stain reagent (Pierce) to ensure equal loading was achieved in each well.

Immunofluorescence

HUVECs were grown on collagen-coated 8-well chamber slides. After treatment with either media alone or 2 hours after PDT (100 ng/ml verteporfin; 2 J/cm² red light, 620–

700 nm), cells were coated with acetone for 20 minutes at –20°C. Cells were then washed with PBS and incubated with 5% skim milk powder in PBS for 30 minutes at 37°C. Cells were washed with PBS and incubated with or without (not shown) rabbit anti-active caspase 3 antibody (Pharmingen) (1:20) for 30 minutes at 37°C. Cells were washed and incubated with 1% normal goat serum for 30 minutes at 37°C. Goat serum (Biosource, Camarillo, CA) was removed and goat anti-rabbit IgG FITC-conjugated antibody (Biosource, Camarillo, CA) (1:100) in 0.1% normal goat serum was added for 30 minutes at 37°C. Wells were coated with 100 μ l of Antifade reagent (Molecular Probes; Eugene, Oregon). Fluorescent images were acquired through a standard FITC filter set (Omega, Brattleboro, VT) using a 16-bit cooled CCD camera (1024 $\times$ 1024 pixels; Photometrics, Tucson, AZ) mounted on 2.5 $\times$ adapter at the bottom port of an Axiovert S100 TV microscope (Zeiss, North York, ON, Canada) and coupled to the signal acquisition and processing software SlideBook (Intelligent Imaging Innovations, Denver, CO). A 63 $\times$ Zeiss objective was used for obtaining detailed pictures.

Figure 1. Altered cytosolic levels of cytochrome c and Bax and activation of multiple caspases during HUVEC apoptosis. **A:** At the indicated times after PDT, whole cell protein extracts or cytosolic (S-100) extracts were obtained and analyzed via SDS-PAGE followed by Western blotting using antibodies against Bax, cytochrome c, or Bid. **B:** Whole cell protein extracts were separated by SDS-PAGE followed by Western blotting using antibodies to different caspases.

Figure 2. Inhibition of HUVEC apoptosis with ZVAD-fmk. **A:** HUVECs were treated with media alone, verteporfin alone, PDT (light-activated verteporfin), or PDT + ZVAD-fmk. DIC microscopy was used to visualize cells 2 hours after treatment. **B:** HUVECs treated with media alone, verteporfin alone, PDT, or PDT + ZVAD-fmk were lysed 2 hours after treatment and proteins were separated by SDS-PAGE followed by Western blotting using antibodies to caspase 3 and PARP. **C:** Untreated (**top**) or PDT-treated (**bottom**) HUVECs were visualized by open field microscopy (**left**; original magnification, $\times 63$) or for active caspase 3 immunofluorescence (**right**; original magnification, $\times 63$).

Results

Altered Cytosolic Levels of Bax and Cytochrome *c* during HUVEC Apoptosis

Western blot analysis of whole cell protein extracts indicated that the overall expression level and molecular mass of Bax and cytochrome *c* did not change, whereas Bid was undetectable by 2 hours after irradiation (Figure 1A). However, cytosolic (S-100) levels of Bax decreased and it was not detectable by 2 hours after treatment. In contrast, cytosolic cytochrome *c* was evident immediately and its levels increased over 2 hours after treatment (Figure 1A). Morphological evidence of apoptosis was not observed before the initial release of cytochrome *c* and decrease in cytosolic Bax (data not shown).

Activation of Caspases 2, 3, 6, 7, 8, and 9 in HUVECs

Caspase 1, 2, 3, 4, 6, 7, 8, 9, and 10 activation were assessed using Western blotting. Processing of caspases, as determined by either the disappearance of the proform of the enzyme and/or detection of one of the

cleavage intermediates or active subunits (caspase 2, 3, 8, and 9 only), was apparent for caspases 2, 3, 6, 7, 8, and 9 within 1 to 2 hours after treatment (Figure 1B). Caspase 1, 4, or 10 processing was not detected (data not shown).

Inhibition of HUVEC Apoptosis with ZVAD-fmk

HUVECs were incubated with a general caspase family inhibitor (ZVAD-fmk) before photoactivation of verteporfin. Differential interference contrast (DIC) microscopy was used to visualize the effect of ZVAD-fmk on cell morphology (Figure 2A). ZVAD-fmk completely blocked the cell shrinkage and membrane blebbing effects that are commonly associated with apoptosis. ZVAD-fmk blocked caspase 3 processing and subsequent PARP cleavage indicating that ZVAD-fmk was effective at interfering with HUVEC apoptosis (Figure 2B). Verteporfin in the absence of light did not induce caspase 3 activation, PARP cleavage, cell shrinkage or membrane blebbing.

Caspase 3 activation was further exemplified using a caspase 3 antibody that recognizes a conformational epitope which is exposed by activation-induced cleavage of caspase 3. Active caspase 3 was only detected in

PDT-treated (apoptotic) HUVECs (Figure 2C). Minimal fluorescence was detected in normal, untreated HUVECs nor in the untreated or PDT-treated HUVEC control slides that did not receive the primary antibody during the immunostaining procedure (data not shown).

Discussion

The present study illustrates several novel aspects of EC apoptosis. EC apoptosis, as induced by photosensitization of verteporfin, did not reduce intracellular levels of Bax; however, cytosolic levels of Bax did decrease, suggesting a cellular redistribution of Bax from a cytosolic form to a membrane-bound form. Previous studies using fluorescent microscopy to study the intracellular migration of Bax in apoptotic kidney epithelial cells demonstrated that Bax may translocate to mitochondria before cell shrinkage or nuclear condensation.¹⁶ Furthermore, Bax has been shown to directly induce cytochrome *c* release in isolated mitochondria.¹⁷ Whether Bax is directly responsible for the induction of cytochrome *c* release in PDT-treated ECs or whether it occurs as a parallel phenomenon requires further elucidation. However, levels of cytosolic cytochrome *c* were shown to increase reciprocally as levels of cytosolic Bax decreased, suggesting there may be a relationship between the two events during EC apoptosis. Another Bcl-2 homolog, Bid, on cleavage into a truncated form (trBid) by caspase 8, induces cytochrome *c* release during Fas or tumor necrosis factor-induced apoptosis.^{18,19} Although we observed Bid cleavage in our system, it occurred after cytochrome *c* release. This discrepancy may be explained by the differential timing of caspase 8 activation between receptor-mediated apoptosis and non-receptor-induced apoptosis. It has been demonstrated that while caspase 8 is the earliest caspase activated during Fas, tumor necrosis factor, or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis, caspase 8 may be activated downstream of cytochrome *c* release in non-receptor-mediated forms of apoptosis.^{13,20} Since Bid is a substrate of caspase 8, cleavage at a later time point during PDT-mediated apoptosis would be anticipated. On cleavage of Bid by caspase 8, it is possible that trBid may then enhance further release of cytochrome *c* from mitochondria.

It has been suggested that once mitochondrial cytochrome *c* is released into the cytosol, the cell may be irreversibly committed to death.⁹ The mechanism by which cytochrome *c* is released into the cytosol is not understood, but may involve the opening of a mitochondrial megachannel known as the mitochondrial permeability transition pore.⁹ In the case of EC apoptosis, this pore may be sensitive to cyclosporin A.⁸ OxLDL, tumor necrosis factor- α , and angiotensin II induce the release of mitochondrial cytochrome *c*, an event which can be inhibited by pretreatment with cyclosporin A.⁸

Our studies confirm observations of other investigators that caspase 3 is activated during EC apoptosis. We further demonstrate that several other caspases participate in the execution of apoptosis downstream of cyto-

chrome *c* release. We clearly demonstrate for the first time the activation of caspases 2, 6, 7, 8, and 9 during HUVEC apoptosis. Biochemical analysis has revealed that caspase 9 may be the first caspase activated following cytochrome *c* release.²¹ Cytochrome *c* is believed to be necessary for Apaf-1-mediated processing of caspase 9, which, in turn, cleaves other downstream executioner caspases.²¹ Caspase 3 is one of the best characterized caspases and has been termed the "central executioner" of apoptosis. On activation, caspase 3 can cleave numerous proteins involved in cell structure, signaling, and repair and is essential for DNA fragmentation.⁴ Caspase 3 may also facilitate the activation of caspase 2.²² Caspase 8 processing, as evidenced by the appearance of 43- and 41-kD caspase 8a/b intermediate cleavage products, is further exemplified by the disappearance of Bid, a known caspase 8 substrate.^{18,19} Activation of caspase 8 downstream of cytochrome *c* release, followed by Bid cleavage, offers a potential positive feedback mechanism to enhance further cytochrome *c* release during drug-induced apoptosis. Caspase 6, its activation evidenced by the disappearance of its proform, may participate in the cleavage of lamins, major constituents of the nuclear envelope.²³ Caspase 7 appears to have a similar role and substrate preference to caspase 3.³

In summary, EC apoptosis is associated with decreased cytosolic levels of Bax, and increased cytosolic levels of cytochrome *c*. The appearance of cytochrome *c* in the cytosol precedes the activation of multiple caspases, which are ultimately responsible for cleaving various structural, functional, and reparative proteins resulting in the manifestation of an apoptotic phenotype. Further understanding of the events that execute the apoptotic process once the cell is committed to death may be applicable to the understanding of the role of apoptosis, or lack thereof, in the pathogenesis and treatment of common vascular disorders.

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