Mitochondrial Release of Apoptosis-Inducing Factor and Cytochrome c During Smooth Muscle Cell Apoptosis

David J. Granville,^{*†} Brigid A. Cassidy,^{*†}
Dietrich O. Ruehlmann,[‡] Jonathan C. Choy,^{*†}
Catherine Brenner,[§] Guido Kroemer,[¶]
Cornelis van Breemen,[‡] Philippe Margaron,[†]
David W. Hunt,[†] and Bruce M. McManus^{*}

From the Department of Pathology and Laboratory Medicine,^{*} University of British Columbia McDonald Research Laboratories/The iCAPTURE Centre, St. Paul's Hospital/Providence Health Care, University of British Columbia, Vancouver, British Columbia, Canada; QLT Inc.,[†] Vancouver, British Columbia, Canada; the Department of Pharmacology and Therapeutics,[‡] University of British Columbia, Vancouver, British Columbia, Canada; the Centre National de la Recherche Scientifique,[§] Université de Technologie de Compiègne, Compiègne, France; and the Centre National de la Recherche Scientifique,[¶] Institut Gustave Roussy, Villejuif, France

Photodynamic therapy (PDT) is under investigation for the treatment of intimal hyperplasia in conditions such as atherosclerosis and restenosis. Although smooth muscle cells (SMCs) may be a key target for treatment, the effects of PDT on these cells are poorly characterized. In the present study, apoptosis was induced in primary human aortic SMCs by the combination of the photosensitizer verteporfin and visible light. After PDT, an increase in mitochondrial cytochrome c (cyt c) and apoptosis-inducing factor (AIF) levels were detected in the cytosol immediately and their levels increased steadily up to 2 hours. Cytosolic levels of the pro-apoptotic Bcl-2 family member Bax decreased reciprocally throughout this period, but this change did not occur before cyt c release. Confocal microscopy revealed a diffuse staining pattern of cyt c within apoptotic cells as compared to a distinct mitochondrial staining in normal cells. AIF translocated from mitochondria to the nucleus during the progression of apoptosis. After cyt c release, caspase-9 and caspase-3 processing was visible by 1 hour and caspase-6, -7, and -8 processing was apparent by 2 hours after PDT. In summary, these results demonstrate for the first time the cellular redistribution of mitochondrial AIF during SMC apoptosis, as well as the early release of cyt c and the subsequent activation of multiple caspases during PDT-induced SMC apoptosis. (*Am J Pathol* 2001, 159:305–311)

Photodynamic therapy (PDT) is a clinically approved treatment for various types of cancer and the ocular condition known as age-related macular degeneration, the leading cause of blindness in the elderly. PDT is also under investigation for the treatment of other ocular disorders as well as atherosclerosis, restenosis, allograft rejection, and autoimmune disorders.¹ PDT catalyzes the formation of reactive oxygen intermediates and rapidly induces apoptosis in a variety of cell types after light irradiation.^{2–5}

In vivo studies have shown that PDT represents a safe, effective method of inhibiting the development of intimal hyperplasia.^{6,7} Furthermore, a PDT-mediated reduction in the number of smooth muscle cells (SMCs) occurs along with the prevention of the inflammatory infiltration, aneurysmal dilatation, and development of intimal hyperplasia associated with allograft rejection.⁸ Although recent studies have suggested that PDT-induced apoptosis plays a primary role in the reduction of intimal hyperplasia in balloon-injured rat carotid arteries,⁹ little is known regarding the biochemical effects of PDT on SMCs.

In addition to acting as the main source of cellular ATP production, mitochondria may also regulate cell death.¹⁰ Since the initial observation that Bcl-2 inhibits apoptosis by preventing mitochondrial cytochrome c (cyt c) release,^{11,12} much research has been devoted to the role of mitochondria in apoptosis. Therefore, the search for chemical agents that directly target mitochondria to induce apoptosis has gained much attention in recent years. Porphyrin-derived photosensitizers may localize to mitochondria.^{13,14} Furthermore, a number of groups have now shown that cyt c is released into the cytosol immediately after photosensitization of various tumor cell lines using different photosensitizers.^{2,14–19}

Apoptosis-inducing factor (AIF) is a recently characterized pro-apoptotic mitochondrial protein.²⁰ Similar to cyt c, AIF is a bifunctional protein with both an electron acceptor/donor (oxidoreductase) function and an apop-

Supported in part by grants from the Heart and Stroke Foundation of British Columbia and Yukon (to B. M. M., C. V. B.), the St. Paul's Hospital Foundation (to B. M. M.) and the Foundation pour la Recherche Médicale (to C. B.).

Accepted for publication March 19, 2001.

Address reprint requests to Bruce McManus, M.D., Ph.D., Department of Pathology and Laboratory Medicine, University of British Columbia–St. Paul's Hospital, 1081 Burrard St., Vancouver, B.C., Canada V6Z 1Y6. E-mail: mcmanus@interchange.ubc.ca.

togenic function.²¹ AIF has been shown in other cell types to be released from mitochondria whereupon it translocates to nuclei and stimulates chromatin condensation and incomplete 50-kb DNA fragmentation referred to as stage I DNA fragmentation.²² This stage of apoptosis is not dependent on caspases (cysteine-specific proteases). Caspases comprise a family of proteases that are responsible for the majority of events pertaining to the execution of the apoptotic program.²³ Complete (stage II) DNA fragmentation into 200-bp fragments requires caspase-3-mediated cleavage of DNA fragmentation factor/inhibitor of caspase activated deoxyribonuclease (DFF/ICAD) that is normally bound to CAD within the cytosol of nonapoptotic cells. Cleavage of DFF/ICAD results in the activation and nuclear translocation of CAD that is responsible for the subsequent cleavage of DNA into 200-kb fragments.^{22,24,25} The involvement of AIF release during PDT-induced apoptosis or during SMC apoptosis in response to any stimuli, to our knowledge, has never been assessed.

The current study demonstrates that mitochondrial events play a crucial role in the initiation of apoptosis in PDT-treated SMCs. Previous studies have indicated that cellular redistribution of the pro-apoptotic Bcl-2 homologue Bax in response to PDT occurs in primary endothelial cells, but not in transformed HeLa cells.^{2,16} These results indicated that individual cell types respond differently to PDT and may use distinct biochemical pathways to achieve apoptotic cell death. In certain systems, Bax has been shown to initiate cyt c release by translocating from the cytosol to mitochondria.^{26,27} In the present study, we demonstrate that cyt c and AIF release occurs before the cellular redistribution of Bax in PDT-treated SMCs suggesting that Bax is not responsible for initiating these events. Furthermore, we define the involvement of multiple caspases in SMC apoptosis.

Materials and Methods

Reagents

Lipid-formulated verteporfin was provided by QLT Inc. (Vancouver, BC, Canada). Antibodies were obtained from the following sources: anti-caspase-6 and -8 (Upstate Biotechnology Inc., Lake Placid, NY); anti-caspase-3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA); anti-caspase-7 (R&D Systems, Minneapolis, MN); anti-native cyt c-clone 6H2.B4 (immunocytochemistry) and anti-denatured cyt c-clone 7H8.2C12 (Western blotting), anti-Bax, anti-caspase-9 (Pharmingen, Mississauga, ON); poly (ADP-ribose) polymerase (Biomol Research Laboratories, Plymouth Meeting, PA). The anti-AIF antibody was generated as previously described.²⁰

Cell Culture and PDT

Human aortic smooth muscle cells (HASMCs) (Clonetics, San Diego, CA) were maintained in smooth-muscle cell basal medium (Clonetics) supplemented with 10% heat-inactivated fetal bovine serum, insulin (5 μ g/ml), hFGF-B

(2 ng/ml), gentamicin (50 μ g/ml), amphotericin B (50 ng/ml), and epidermal growth factor (0.5 ng/ml) (Clonetics). HASMCs were grown on 10-cm Petri dishes until they were 80 to 90% confluent. Cells were incubated for 60 minutes in the dark at 37°C with or without verteporfin (0 to 100 ng/ml) in smooth-muscle cell basal medium supplemented with 2% fetal bovine serum. After drug incubation, HASMCs were exposed to red fluorescent light (620 to 700 nm) delivered at a rate of 5.6 mW/cm² to give a total dose of 2 J/cm².

Analysis of DNA Status

The propidium iodide fluorescence analysis procedure was used to detect changes in the status of cellular DNA.^{28,29} At 0 to 5 hours after PDT, 1×10^6 cells were scraped off of the plates, washed twice with ice-cold phosphate-buffered saline (PBS), then permeabilized and fixed in 80% ethanol at 4°C for 1 hour. Cells were washed twice in ice-cold PBS and treated with RNase (5 U/ml, DNase-free) and stained with propidium iodide (50 μ g/ml) in PBS. Samples were analyzed by flow cytometry. The percentage of cells containing hypodiploid levels of DNA was calculated from single-parameter flow cytometry for propidium iodide fluorescence²⁸ using an Epics XL flow cytometer (Coulter Electronics Inc., Hialeah, FL).

Caspase Activity and Immunoblot Analysis

Whole and cytosolic (S-100) cell extracts were prepared and Western blotting was performed as previously described.² Whole cell lysates were assessed for caspase-3/7-like (DEVD-ase) activity as previously described.²

Immunofluorescence

HASMCs were grown on type I collagen (VWR Canlab, Mississauga, ON, Canada)-coated 8-well chamber slides. After treatment with media alone or PDT, cells were coated with 2% paraformaldehyde for 15 minutes at room temperature. Cells were washed with PBS and incubated with or without mouse anti-cyt, rabbit anti-AIF, or mouse anti-Bax monoclonal antibodies (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-mouse IgG Alexa 488-conjugated or goat anti-rabbit IgG Alexa 488 antibody (Molecular Probes, Eugene, OR) (1:200) 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). Fluorescence images for Bax and cyt c were acquired using a laser-scanning confocal system (Noran OZ, Bicester, UK) on an inverted microscope equipped with a $\times 100$ oil-immersion lens. The cells were illuminated using the 488-nm line of an argon-krypton laser. Image analysis was performed in ImagePro Plus and all data were stored on CD-ROM. For AIF, fluorescent images were acquired through a standard fluorescein-isothiocyanate filter set (Omega Optical Inc., Brattleboro, VT) using a 16-bit cooled charge-cou-

pled device camera (1024 × 1024 pixels; Photometrics, AZ) mounted on ×2.5 adaptor at the bottom port of an Axiovert S100 TV microscope (Zeiss, Thornwood, NY) and coupled to the signal acquisition and processing software SlideBook (Intelligent Imaging Innovations, Denver, CO). A ×40 Zeiss objective was used for obtaining the AIF photomicrographs.

Mitochondrial Epitope 7A6 Antigen Expression

Detection of the mitochondrial epitope 7A6 by the mouse monoclonal Apo2.7 antibody has been previously used as a tool to measure apoptosis.³⁰ HASMCs were prepared for flow cytometry by washing twice with PBS followed by a 5-minute incubation at 37°C in PBS containing 0.05% trypsin and 0.53 mmol/L ethylenediaminetetraacetic acid. Mitochondrial 7A6 antigen expression by apoptotic HASMCs was assessed using flow cytometry as described.¹⁶

Results

Cellular Redistribution of Bax, Cyt c, and AIF During HASMC Apoptosis

Analysis of whole cell extracts by Western blotting indicated that the overall levels or the molecular mass of Bax, cyt c, and AIF (Figure 1A) were not altered throughout the 2-hour time period after PDT. However, a decrease in cytosolic levels of Bax was observed and Bax became minimally detectable by 2 hours after PDT (Figure 1A). Bax immunostaining exhibited a more punctate cellular distribution in apoptotic HASMCs (Figure 1B) in agreement with Western blot data indicating a cellular redistribution of Bax during PDT-induced apoptosis. By 2 hours after PDT, cell shrinkage had advanced to such an extent that cellular localization of Bax was impossible.

In contrast to Bax, cyt c was not present in the cytosolic fraction of untreated cell lysates, but was evident in lysates prepared immediately after PDT, increasing steadily up to 2 hours after treatment (Figure 1A). Confocal microscopy revealed a very pronounced, string-like staining pattern for cyt c in untreated cells but a more diffuse staining in PDT-treated cells (Figure 1B). To determine the timing of mitochondrial 7A6 antigen unmasking, the monoclonal Apo2.7 antibody followed by flow cytometry was used at times corresponding to those for cyt c, AIF, and Bax assays (Figure 1C). Increased 7A6 expression corresponded with the increase in cytosolic cyt c levels after PDT.

Correspondent with results for cyt c, cytosolic levels of AIF were detected immediately and increased throughout the 2-hour period after PDT (Figure 1A). Furthermore, when assessed using indirect immunofluorescence microscopy, we observed a redistribution of AIF from mitochondria to the nucleus (Figure 1B).

Caspase-3, -6, -7, -8, and -9 Activation and DNA Fragmentation During PDT-Induced HASMC Apoptosis

Processing of caspases, as determined by either the disappearance of the proform of the enzyme (caspase-6) and/or appearance of one or more of the active subunits (caspase-3, -7, -8, and -9), was apparent for all caspases tested and these changes increased in a time-dependent manner after PDT (Figure 2A). Processing of caspase-9 and incomplete processing of caspase-3 (19-kd band) were visible by 1 hour after PDT. Complete caspase-3 processing (17-kd band) and caspase-6, -7, and -8 processing were not detected until 2 hours after PDT. Poly (ADP-ribose) polymerase cleavage was also not detected until 2 hours after PDT. The multicaspase inhibitor ZVAD-fmk inhibited caspase activity associated with PDT (Figure 2B), although mitochondrial 7A6 expression was not altered in the presence of the peptide (Figure 2C). Cells treated with verteporfin in the absence of light did not induce caspase activity (not shown). An increase in DNA fragmentation was observed by 1 hour and >40% of cells exhibited DNA fragmentation by 5 hours after PDT (Figure 2D).

Discussion

It is becoming clear that altered regulation or absence of apoptosis may contribute to the pathogenesis of many common cardiovascular conditions.^{31–34} The biochemical mechanisms related to SMC apoptosis, in response to any stimulus, are poorly defined. PDT is under investigation for the treatment of intimal hyperplasia associated with restenosis and atherosclerosis. Because SMCs may be an important target for PDT in the reduction of intimal hyperplasia, it is important to understand the mechanism by which PDT affects these cells.

In the current study, PDT was found to elicit the immediate release of cyt c from mitochondria. Furthermore, using Western blotting, which is more sensitive than immunofluorescence, levels of cyt c were present in the cytosol immediately after photosensitization. It is highly unlikely that Bax is the primary stimulus for initiating cyt c release. Such an effect would require almost instantaneous Bax translocation and membrane insertion into mitochondria to release cyt c immediately after PDT. Additionally, the cytosolic levels of Bax did not seem to be altered immediately after PDT indicating that cellular redistribution of Bax from a cytosolic to a membrane-bound form had not occurred at this stage. Whether other Bcl-2 family members regulate cyt c release in SMCs in response to PDT requires further elucidation, however it is more likely that PDT has a direct effect on mitochondria. In support of the latter concept, porphyrin-derived photosensitizers may localize to and directly bind mitochondrial proteins such as the peripheral benzodiazepine receptor or the adenine nucleotide translocator.^{35,36} Thus, it is most likely that PDT-induced SMC apoptosis is triggered at or near mitochondria.

Figure 2. Activation of multiple caspases during HASMC apoptosis. **A:** Untreated or PDT-treated HASMCs at 0, 1, or 2 hours after treatment were lysed and assessed by Western blotting for the status of caspase-3, -6, -7, -8, or -9. **Arrows** point to cleavage fragments detected with each antibody. **B:** Cells were pretreated with ZVAD-fmk for 30 minutes before photosensitization. Cell extracts were assessed for caspase-3/7-like (DEVDase) activity (**top**) or caspase-3 processing using Western blotting (**bottom**). **C:** Cells were treated with ZVAD-fmk for 30 minutes before photosensitization. Expression of the mitochondrial 7A6 antigen was assessed using the monoclonal Apo2.7 antibody and flow cytometry as described. Isotype controls are shown in **shaded histograms**. **D:** Induction of DNA fragmentation in HASMCs after PDT. PDT-treated HASMCs were assessed at the indicated times after PDT. Controls (medium or verteporfin) were assessed after 5 hours. Bars represent mean values for three experiments $\pm$ SD.

Further evidence of PDT acting on mitochondria is provided by the presence of the mitochondrial 7A6 antigen after PDT and release of AIF. AIF has recently been shown to play a key role in the regulation of stage I nuclear apoptosis consisting of chromatin condensation and 50-kb DNA fragmentation.²² During apoptosis, AIF is

released from mitochondria whereupon it translocates to the nucleus and initiates these events. In the current set of experiments using SMCs, increased AIF was detected in the cytosol after PDT indicating that it had been released from mitochondria. Furthermore, AIF exhibited a staining pattern that would support the concept of AIF

translocation to the nucleus. To our knowledge, these results are the first to document a role for AIF in SMC apoptosis in response to any stimulus and imply a role for AIF during PDT-induced apoptosis.

In addition to mitochondria-associated apoptotic events, the involvement of caspases, the central executioner molecules of apoptosis, were assessed. Processing of caspases-3, -6, -7, -8, and -9 followed the appearance of cyt c in the cytosol. Although certain caspases may perform redundant functions, activation of multiple caspases is likely to result in amplification of cyt c release and further caspase activity, in addition to the cleavage of specific structural, signaling, and reparative proteins leading to an efficient disassembly of the cell. In the current system, caspase-8 was clearly activated downstream of cyt c and AIF release indicating that it is not the primary instigator of cyt c release. It is likely that caspase-3 and caspase-8 activation downstream of cyt c serves as a feedback loop to amplify further cyt c release via the processing of Bid into its truncated form that is capable of inducing cyt c release.^{37,38} Future investigations will focus on the significance of this and other feedback mechanisms that may serve to enhance cyt c release.

In summary, PDT induces SMC apoptosis, a process that involves the cellular redistribution of Bax, AIF, and cyt c. Because of the rapidity by which AIF and cyt c are released from mitochondria after photosensitization, it is unlikely that Bax is the primary stimulus for initiating their release. Furthermore, translocation of AIF from mitochondria to the nucleus does seem to play a role in SMC apoptosis. Results from the current study not only provide insight into a possible mechanism for PDT-mediated reduction of SMCs in treatment of vascular lesions, but they also are applicable to a broader understanding of SMC injury and how these cells respond to cytotoxic stimuli.

References

- Levy JG: Photodynamic therapy. *Trends Biotechnol* 1995, 13:14–18
- Granville DJ, Shaw JR, Leong S, Carthy CM, Margaron P, Hunt DW, McManus BM: Release of cytochrome c, Bax migration, Bid cleavage, and activation of caspases 2, 3, 6, 7, 8, and 9 during endothelial cell apoptosis. *Am J Pathol* 1999, 155:1021–1025
- Granville DJ, Carthy CM, Jiang H, Levy JG, McManus BM, Matroule J, Piette J, Hunt DW: Nuclear factor- κ B activation by the photochemotherapeutic agent verteporfin. *Blood* 2000, 95:256–262
- Granville DJ, Jiang H, An MT, Levy JG, McManus BM, Hunt DW: Bcl-2 overexpression blocks caspase activation and downstream apoptotic events instigated by photodynamic therapy. *Br J Cancer* 1999, 79:95–100
- Jiang H, Granville DJ, McManus BM, Levy JG, Hunt DW: Selective depletion of a thymocyte subset in vitro with an immunomodulatory photosensitizer. *Clin Immunol* 1999, 91:178–187
- Adili F, Status van Eps RG, Flotte TJ, LaMuraglia GM: Photodynamic therapy with local photosensitizer delivery inhibits experimental intimal hyperplasia. *Lasers Surg Med* 1998, 23:263–273
- Rockson SG, Lorenz DP, Cheong WF, Woodburn KW: Photoangioplasty: an emerging clinical cardiovascular role for photodynamic therapy. *Circulation* 2000, 102:591–596
- LaMuraglia GM, Adili F, Schmitz-Rixen T, Michaud NA, Flotte TJ: Photodynamic therapy inhibits experimental allograft rejection. A novel approach for the development of vascular bioprostheses. *Circulation* 1995, 92:1919–1926
- LaMuraglia GM, Schiereck J, Heckenkamp J, Nigri G, Waterman P, Leszczynski D, Kossodo S: Photodynamic therapy induces apoptosis in intimal hyperplastic arteries. *Am J Pathol* 2000, 157:867–875
- Green DR, Reed JC: Mitochondria and apoptosis. *Science* 1998, 281:1309–1312
- Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD: The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 1997, 275:1132–1136
- Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng T, Jones DP, Wang X: Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* 1997, 275:1129–1132
- Kessel D, Luo Y, Deng Y, Chang CK: The role of subcellular localization in initiation of apoptosis by photodynamic therapy. *Photochem Photobiol* 1997, 65:422–426
- Kessel D, Luo Y: Photodynamic therapy: a mitochondrial inducer of apoptosis. *Cell Death Differ* 1999, 6:28–35
- Granville DJ, Carthy CM, Jiang H, Shore GC, McManus BM, Hunt DW: Rapid cytochrome c release, activation of caspases 3, 6, 7 and 8 followed by Bap31 cleavage in HeLa cells treated with photodynamic therapy. *FEBS Lett* 1998, 437:5–10
- Carthy CM, Granville DJ, Jiang H, Levy JG, Rudin CM, Thompson CB, McManus BM, Hunt DW: Early release of mitochondrial cytochrome c and expression of mitochondrial epitope 7A6 with a porphyrin-derived photosensitizer: bcl-2 and Bcl-xL overexpression do not prevent early mitochondrial events but still depress caspase activity. *Lab Invest* 1999, 79:953–965
- Inanami O, Yoshito A, Takahashi K, Hiraoka W, Kuwabara M: Effects of BAPTA-AM and forskolin on apoptosis and cytochrome c release in photosensitized Chinese hamster V79 cells. *Photochem Photobiol* 1999, 70:650–655
- Varnes ME, Chiu SM, Xue LY, Oleinick NL: Photodynamic therapy-induced apoptosis in lymphoma cells: translocation of cytochrome c causes inhibition of respiration as well as caspase activation. *Biochem Biophys Res Commun* 1999, 255:673–679
- Vantighem A, Assefa Z, Vandenabeele P, Declercq W, Courtois S, Vandenheede JR, Merlevede W, de Witte P, Agostinis P: Hypericin-induced photosensitization of HeLa cells leads to apoptosis or necrosis. Involvement of cytochrome c and procaspase-3 activation in the mechanism of apoptosis. *FEBS Lett* 1998, 440:19–24
- Susin SA, Lorenzo HK, Zamzami N, Marzo I, Snow BE, Brothers GM, Mangion J, Jacotot E, Costantini P, Loeffler M, Larochette N, Goodlett DR, Aebersold R, Siderovski DP, Penninger JM, Kroemer G: Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* 1999, 397:441–446
- Daugas E, Nochy D, Ravagnan L, Loeffler M, Susin SA, Zamzami N, Kroemer G: Apoptosis-inducing factor (AIF): a ubiquitous mitochondrial oxidoreductase involved in apoptosis. *FEBS Lett* 2000, 476:118–123
- Susin SA, Daugas E, Ravagnan L, Samejima K, Zamzami N, Loeffler M, Costantini P, Ferri KF, Irinopoulou T, Prevost MC, Brothers G, Mak TW, Penninger J, Earnshaw WC, Kroemer G: Two distinct pathways leading to nuclear apoptosis. *J Exp Med* 2000, 192:571–580
- Nicholson DW: Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Differ* 1999, 6:1028–1042
- Liu X, Zou H, Slaughter C, Wang X: DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell* 1997, 89:175–184
- Sakahira H, Enari M, Nagata S: Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* 1998, 391:96–99
- Wolter KG, Hsu YT, Smith CL, Nechushtan A, Xi XG, Youle RJ: Movement of bax from the cytosol to mitochondria during apoptosis. *J Cell Biol* 1997, 139:1281–1292
- Hsu YT, Wolter KG, Youle RJ: Cytosol-to-membrane redistribution of Bax and Bcl-XL during apoptosis. *Proc Natl Acad Sci USA* 1997, 94:3668–3672
- Telford WG, King LE, Fraker PJ: Rapid quantitation of apoptosis in pure and heterogeneous cell populations using flow cytometry. *J Immunol Methods* 1994, 172:1–16

29. Darzynkiewicz Z, Bruno S, Del Bino G, Gorczyca W, Hotz M, Lassota P, Traganos F: Features of apoptotic cells measured by flow cytometry. *Cytometry* 1992, 13:795–808
30. Koester SK, Roth P, Mikulka WR, Schlossman SF, Zhang C, Bolton WE: Monitoring early cellular responses in apoptosis is aided by the mitochondrial membrane protein-specific monoclonal antibody APO2.7. *Cytometry* 1997, 29:306–312
31. Sabbah HN, Sharov VG, Goldstein S: Programmed cell death in the progression of heart failure. *Ann Med* 1998, 30(Suppl 1):S33–S38
32. Best PJ, Hasdai D, Sangiorgi G, Schwartz RS, Holmes Jr DR, Simari RD, Lerman A: Apoptosis. Basic concepts and implications in coronary artery disease. *Arterioscler Thromb Vasc Biol* 1999, 19:14–22
33. Walsh K, Isner JM: Apoptosis in inflammatory-fibroproliferative disorders of the vessel wall. *Cardiovasc Res* 2000, 45:756–765
34. McDonald PC, Wong D, Granville DJ, McManus BM: Emerging roles of endothelial cells and smooth muscle cells in transplant vascular disease. *Transplant Rev* 1999, 13:109–127
35. Verma A, Nye JS, Snyder SH: Porphyrins are endogenous ligands for the mitochondrial (peripheral-type) benzodiazepine receptor. *Proc Natl Acad Sci USA* 1987, 84:2256–2260
36. Belzacq AS, Jacotot E, Vieira HL, Mistro D, Granville DJ, Xie Z, Reed JC, Kroemer G, Brenner C: Apoptosis induction by the photosensitizer verteporfin. Identification of mitochondrial adenine nucleotide translocator as a critical target. *Cancer Res* 2001, 61:1260–1264
37. Slee EA, Keogh SA, Martin SJ: Cleavage of BID during cytotoxic drug and UV radiation-induced apoptosis occurs downstream of the point of Bcl-2 action and is catalysed by caspase-3: a potential feedback loop for amplification of apoptosis-associated mitochondrial cytochrome c release. *Cell Death Differ* 2000, 7:556–565
38. Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD, Wang HG, Reed JC, Nicholson DW, Alnemri ES, Green DR, Martin SJ: Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. *J Cell Biol* 1999, 144:281–292