Induction of Apoptosis by Pyrrolidinedithiocarbamate and N-Acetylcysteine in Vascular Smooth Muscle Cells*

J er-Chia Tsaï, Mukesh J aint, Chung-Ming Hsieh, Wen-Sen L eet, Masao Yoshizumi, Cam Patterson, Mark A. Perrellat, Carol Cooke, Hong Wang, Edgar Habert, Robert Schlegel, and Mu-En Leet

From the Cardiovascular Biology Laboratory and the Department of Molecular and Cellular Toxicology, Harvard School of Public Health, the Department of Medicine, Harvard Medical School, and the Human Frontier Science Program.


tion in arteriosclerotic lesions. The use of antioxidants to induce apoptosis in vascular smooth muscle cells has been unclear. Pyrrolidinedithiocarbamate (PDTC) and N-acetylcysteine (NAC) have been used as antioxidants to prevent apoptosis in lymphocytes, neurons, and vascular endothelial cells. We report here that PDTC and NAC induce apoptosis in rat and human smooth muscle cells. In rat aortic smooth muscle cells, PDTC induced cell shrinkage, chromatin condensation, and DNA strand breaks consistent with apoptosis. In addition, overexpression of Bcl-2 suppressed vascular smooth muscle cell death caused by PDTC and NAC. The viability of rat aortic smooth muscle cells decreased within 3 h of treatment with PDTC and was reduced to 30% at 12 h. The effect of PDTC and NAC on smooth muscle cells was not species specific because PDTC and NAC both caused dose-dependent reductions in viability in rat and human aortic smooth muscle cells. In contrast, neither PDTC nor NAC reduced viability in human aortic endothelial cells. The use of antioxidants to induce apoptosis in vascular smooth muscle cells may help prevent their proliferation in arteriosclerotic lesions.

Apoptosis or programmed cell death is characterized by cell shrinkage, membrane blebbing, and chromatin condensation that culminate in cell fragmentation (1). Stimuli as diverse as hyperthermia, growth factor withdrawal, chemotherapeutic agents, radiation, and oxidative stress induce apoptosis in many cell types (2–5), and several cellular proteins have been identified that activate or suppress it (6–13). The B-cell leukemia/lymphoma-2 protein (Bcl-2) has been shown to prevent apoptosis induced by diverse stimuli (14–17), perhaps by acting as an antioxidant (14, 18). This hypothesis is consistent with observations that antioxidants such as pyrrolidinedithiocarbamate (PDTC) and N-acetylcysteine (NAC) prevent apoptosis in lymphocytes (14, 19–21), neurons (18, 22), and vascular endothelial cells (23).

Proliferation of vascular smooth muscle cells is one of the most important features of arteriosclerosis (24). Rao and Berk (25) have shown that hydrogen peroxide stimulates proliferation of vascular smooth muscle cells but inhibits proliferation of vascular endothelial cells. However, the effect of antioxidants on smooth muscle cells has been unclear. PDTC and NAC are two structurally different thio-containing agents. Although NAC at low concentrations (~1 mM) has been reported to cause oxidative stress (5), both agents have been shown to function as effective antioxidants and to prevent oxidant-induced apoptosis (14, 19–21) and activation of the transcription factors NF-kappa B and AP-1 (26–28) and vascular cell adhesion molecule-1 (29). In this study, we tested the effects of PDTC and NAC on vascular smooth muscle and endothelial cells and show that both agents, in dose ranges at which they are used as antioxidants, induced apoptosis in rat and human aortic smooth muscle cells but not in human aortic endothelial cells. This induction of apoptosis in smooth muscle cells occurred in a dose- and time-dependent manner. Furthermore, overexpression of Bcl-2 blocked PDTC- and NAC-induced apoptosis in rat aortic smooth muscle cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Rat aortic smooth muscle cells (RASMC) were harvested from male Sprague-Dawley rats (200–250 g) by enzymatic dissociation according to the method of Gunther et al. (30) with modification (31). Fetal RASMC (A7r5) were obtained from ATCC. RASMC were cultured in Dulbecco's modified Eagle's medium (JRH Biosciences, Lenexa, KS) and supplemented with 10% fetal calf serum (HyClone, Logan UT), penicillin (100 units/ml), streptomycin (100 μg/ml), and 25 mM Hepes (pH 7.4) (Sigma). Human aortic smooth muscle cells (HASMC) and human aortic endothelial cells (HAEC) were purchased from Clonetics Corp. (San Diego). HASMC were cultured in MCDB 131 medium supplemented with 5% fetal calf serum, 10 ng/ml recombinant human epidermal growth factor, 2 ng/ml basic fibroblast growth factor, and 5 μg/ml insulin. HAEC were also grown in MCDB 131 medium supplemented with 2% fetal calf serum, 10 ng/ml epidermal growth factor, 12 μg/ml bovine brain extract, and 10 μg/ml heparin.

PC-12 cells were obtained from ATCC and cultured in RPMI 1640 medium (Mediatech, Washington D.C.) supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum (32). Cells were passaged every 3–5 days, and experiments were performed on cells five to eight passages from primary culture. We purchased PDTC, deferoxamine, penicillamine, and bathocuproinedisulfonic acid (BPSA) from Sigma; NAC (L form) from Chiron Therapeutics (Emeryville, CA); Hoechst 33258 from Molecular Probes Inc.; terminal deoxynucleotidyl transferase (TdT) and biotinylated dUTP from Boehringer Mannheim; and anti-human Bcl-2 polyclonal antibody from Pharmingen (San Diego, CA).

Induction and Morphologic Study of Apoptosis—PDTC and NAC, at various concentrations, were added to subconfluent and exponentially growing cultures of PC-12 cells.
Apoptosis in Aortic Smooth Muscle Cells

growing RASMC, HASMC, and HAEC. Apoptotic cells were identified by inverted light, fluorescence, and electron microscopy. For fluorescence microscopy, cells grown on coverslips were washed with Dulbecco’s phosphate-buffered saline and then fixed in 4% paraformaldehyde in Dulbecco’s phosphate-buffered saline. After fixation, the cells were stained for 10 min with Hoechst 33258 at 8 μg/ml. Cells containing apoptotic nuclei were examined by fluorescence microscopy at 600× magnification. For electron microscopy, cells in 60-mm Petri dishes were fixed in 2% glutaraldehyde in Dulbecco’s phosphate-buffered saline for 30 min at room temperature followed by fixation with 1% osmium tetroxide in 0.1 M cacodylate buffer for 30 min at room temperature, essentially as described (33). TdT-mediated dUTP-biotin nick end labeling was used to detect DNA breaks in apoptotic cells in situ (34). Cells grown on coverslips were rinsed three times with phosphate-buffered saline, incubated in avidin (25 μg/ml in phosphate-buffered saline and 0.4% Triton-X 100) for 30 min at room temperature to block endogenous biotin, rinsed three times with phosphate-buffered saline, and then incubated in 3% H2O2 at room temperature for 10 min. After another three rinses with phosphate-buffered saline, the cells were rinsed with TdT buffer and incubated in 50 μl of TdT buffer (10 units of TdT and 0.5 nmol of biotinylated dUTP) at 37 °C for 60 min. Biotinylated dUTP incorporated into DNA breaks in the nuclei was detected by an avidin-biotin complex method (DAB/nickel chromogen). RASMC were counterstained with eosin.

Viability Assay—Subconfluent, exponentially growing RASMC, HASMC, and PC-12 cells in 24-well plates were incubated with PDTC or NAC for the indicated times. Cell viability was determined by a modified 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, which is based on the conversion of the tetrazolium salt 3-(4,5-dimethyl thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)H-tetrazolium by mitochondrial dehydrogenase to a formazan product (35), as measured at an absorbance of 490 nm.

RESULTS AND DISCUSSION

After 6 h of exposure to 150 μM PDTC, normal RASMC (Fig. 1A) underwent cell shrinkage characteristic of apoptosis (Fig. 1B); fluorescent staining of the DNA revealed chromatin condensation in PDTC-treated RASMC (Fig. 1D) but not in untreated RASMC (Fig. 1C). Another hallmark of apoptosis is DNA strand breaks caused by endonuclease, which can be detected in situ by nick end labeling tissue sections with dUTP-biotin by terminal deoxynucleotidyl transferase (34, 38). In contrast with untreated cells (Fig. 1E), positive staining was visible in most of the nuclei in RASMC that had been treated with PDTC (Fig. 1F). Finally, electron microscopy revealed highly condensed chromatin localized to the inner side of an intact nuclear membrane in PDTC-treated RASMC. The state of the treated nucleus (Fig. 1H) was in sharp contrast with that of the normal (untreated) nucleus (Fig. 1G). In addition to being a thio-containing antioxidant, PDTC is a metal chelator. To exclude the possibility that the effect of PDTC depended solely on its ability to chelate metals, we treated RASMC with NAC. NAC is another thio-containing antioxidant that does not have the ability to chelate metals. RASMC treated with 10 mM NAC manifested morphologic changes identical to those observed in PDTC-treated RASMC (not shown), indicating that two different antioxidants induce apoptosis in vascular smooth muscle cells.

We also used a modified MTT assay of cell viability (35, 39) to measure antioxidant-induced apoptosis. The viability of RASMC decreased within 3 h of treatment with PDTC (Fig. 2A) and was reduced to approximately 30% of base line at 12 h. PDTC also decreased the viability of RASMC in a dose-dependent manner (Fig. 2B). As little as 25 μM PDTC reduced rat aortic smooth muscle cell viability by 25%, whereas 150 μM PDTC reduced viability by 73%. This decrease in vascular smooth muscle cell survival was not specific to rats; PDTC (Fig. 2C) and NAC (Fig. 2D) both caused dose-dependent reductions in survival in HASMC at 24 h. In contrast, neither PDTC nor NAC reduced survival in HAEC (Fig. 2, C and D).

The concentrations of NAC that induced apoptosis in vascular smooth muscle cells (Fig. 2D) have been shown to prevent
apoptosis in lymphocytes, neurons, and endothelial cells (14, 19–21). To confirm that an antioxidant could prevent apoptosis under our culture conditions, we performed experiments in PC-12 neuronal cells. Serum deprivation induced apoptosis in PC-12 cells (Fig. 3), as Greene (40) has also shown. PDTC inhibited this apoptosis in a dose-dependent manner (Fig. 3), and 100 μM PDTC completely prevented apoptosis in PC-12 cells. This observation suggests that the induction of apoptosis by antioxidants in RASMCS and HASMC is cell type specific.

To determine whether Bcl-2 inhibited antioxidant-induced apoptosis in vascular smooth muscle cells, we transfected into fetal RASMCS expression plasmids that did or did not contain the human Bcl-2 coding region. Several stably transfected clones were isolated, and Bcl-2 expression was confirmed by Western blotting with an antibody against human Bcl-2 (Fig. 4A). As in adult RASMCS (Fig. 2A), PDTC (Fig. 4B) and NAC (not shown) both induced dose-dependent apoptosis in fetal RASMCS. Cells that overexpressed Bcl-2, however, were resistant to apoptosis induced by PDTC (Fig. 4B) and NAC (data not shown).

Reactive oxygen species (superoxide anion (·O2−), hydroxyl radical (OH·), and hydrogen peroxide (H2O2)) have been implicated in causing cell damage and cell death (14, 18, 41). Yet, we have observed that PDTC and NAC, two structurally different antioxidants that prevent apoptosis in other cell types in vitro, induced apoptosis in human and rat aortic smooth muscle cells (Figs. 1 and 2). The unique susceptibility to antioxidants of vascular smooth muscle cells indicates that they respond differently than other cell types to changes in the reduction-oxidation state. Consistent with this view, Rao and Berk (25) have shown that H2O2 increases proliferation of vascular smooth muscle cells but inhibits proliferation of vascular endothelial cells.

In the presence of iron or copper, antioxidants such as ascorbic acid act as prooxidants via the Fenton reaction (42, 43). To rule out the possibility that the antioxidant-induced apoptosis we observed was caused simply by autooxidation and subsequent generation of free radicals, we treated RASMCS with PDTC in the presence or absence of 50 μM deferoxamine, d-penicillamine, or BPSA (this concentration was chosen because it was sufficient to allow chelation of 100 times the iron (deferoxamine) or copper (d-penicillamine and BPSA) actually present in the culture medium). Neither deferoxamine nor d-penicillamine affected PDTC-induced apoptosis, and BPSA prevented only 20% of PDTC-induced apoptosis. Thus, the antioxidant-induced apoptosis we observed in RASMCS cannot be ascribed simply to autooxidation by the thio compounds added during the experiment.

Although the dose-response curve for the PDTC-induced decrease in RASMCS survival is rather linear (Fig. 2B), this pattern is not observed universally for antioxidants in other cell types. For example, McCord and co-workers (44–46) published a bell-shaped dose-response curve for the protective effect of superoxide dismutase against ischemia-reperfusion injury in rat and rabbit hearts. High doses of superoxide dismutase were less effective than low doses, and very high doses had a deleterious effect.

Bcl-2 has been shown to protect cell types of diverse lineage from apoptosis induced by many stimuli (14–17). Our finding that Bcl-2 suppresses antioxidant-induced cell death (Fig. 4B) supports our conclusion from morphological studies that vascular smooth muscle cell death caused by PDTC and NAC is apoptosis. Bcl-2 has been shown to prevent apoptosis in lymphocytes and neurons by regulating an antioxidant pathway (14, 18). However, Bcl-2 has also been shown to function as a prooxidant (47). Furthermore, recent reports indicate that Bcl-2 prevents apoptosis induced by hypoxia (48) or by staurosporine under very low oxygen conditions (49). Thus, Bcl-2 may also prevent apoptosis via a mechanism unrelated to its effect on reactive oxygen species. Our observation that Bcl-2 overexpression rescues RASMCS from apoptosis induced by PDTC (Fig. 4B) and NAC also suggests that Bcl-2 may prevent apo-
vascular endothelial cells, may provide a new therapeutic
treatment for transplantation arteriosclerosis. Our finding that antioxidants pro-
tected against PDTC-induced apoptosis in vascular smooth muscle cells, but not in
peripheral endothelial cells. PDTC treatment of fetal RASMC (A7r5 cells, ATCC) with a Bcl-2 expression
plasmid (36) or control plasmid. Clones were selected in medium containing
PDTC. Because fetal RASMC are very sensitive to antioxidants, they
were treated with 25–75 μM PDTC. Viability was determined by
clonogenic assay. PDTC reduced the viability of control RASMC (black bars) in a dose-dependent manner. Viability in the two lines of RASMC
that overexpressed Bcl-2 (△△) in a dose-dependent manner was
significantly greater than in the control line. * p < 0.05, treated versus untreated control. $ p < 0.05, Bcl-2 groups versus control.

ptosis in vascular smooth muscle cells through a pathway unrelated to its antioxidant activity.

Arteriosclerosis and its complications, heart attack and stroke, are the major causes of death in developed countries
(24). Since proliferation of vascular smooth muscle cells is one of the key features of arteriosclerosis, restenosis
after balloon angioplasty or coronary bypass surgery, and transplant arteriosclerosis), our finding that antioxidants pro-
mote apoptosis in vascular smooth muscle cells, but not in
vascular endothelial cells, may provide a new therapeutic strategy for the treatment of arteriosclerosis.

Acknowledgments—We thank Dr. Y. Tsujimoto for the Bcl-2 plasmid, B. Ith for technical assistance, and T. McVarish for editorial assistance.

REFERENCES
1. Kerr, J. F. R., Wyllie, A. H., and Currie, A. R. (1972) Br. J. Canc. 26, 239–257
2. Cohen, J. J. (1994) J. Lab. Clin. Med. 124, 761–765
3. Gershenson, L. E., and Rotello, R. J. (1992) FASEB J. 6, 2450–2455
4. Lin, K.-T., Xue, J.-Y., Nomen, M., Spur, B., and Wong, P. Y.-K. (1995) J. Biol. Chem. 270, 16487–16490
5. Morse, N. R., Tebby, P. W., Sandstrom, P. A., and Burke, T. M. (1995) Protoplasma 184, 184–181
6. Williams, G. T., and Smith, C. A. (1993) Cell 74, 777–779
7. Oliva, Z. N., and Korsmeyer, S. J. (1994) Cell 79, 189–192
8. White, E. (1993) Genes & Dev. 7, 2277–2284
9. Barinaga, M. (1994) Science 263, 754–756
10. Raff, M. C. (1994) Science 264, 668–669
11. Farrow, S. N., White, J. H., Martinou, I., Raven, T., Pun, K. T., Grinham, C. J.,
   Martinou, J. C., and Brown, R. (1995) Nature 374, 731–733
12. Chittenden, T., Harrington, E. A., O’Connor, R., Flemington, C., Lutz, R. J.,
   Evans, G. I., and Guild, B. C. (1995) Nature 374, 733–736
13. Kiefer, M. C., Brauer, M. J., Powers, V. C., Wu, J. J., Umansky, S. R., Tone, L. D., and Barr, P. J. (1995) Nature 374, 736–739
14. Hockenberg, D. M., Oliva, Z. N., Yin, X. M., Milliman, C. L., and Korsmeyer, S. J. (1993) Cell 75, 241–251
15. Jacobson, M. D., Burns, J. F., King, M. P., Miyashita, T., Reed, J. C., and Raff, M. C. (1993) Nature 365, 365–369
16. Vaux, D. L., Haedeker, G., and Stass, A. (1994) Cell 75, 777–779
17. Garcia, I., Martinou, I., Tsujimoto, Y., and Martinou, J. C. (1992) Science 258, 302–304
18. Dancey, J., and Llewellyn-Jones, B. J. (1992) Lancet ii, 233–240
19. Pan, J., and Sawa, K. (1992) J. Cell Biol. 124, 2247–2258
20. Frade, R., Scherk, R., and Baeuerle, P. A. (1992) FASEB J. 6, 2477–2485
21. Schenk, H., Klein, M., Erdbrugger, W., Droge, W., and Schulze-Osthoff, K. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 1672–1676
22. Schenk, R., Rieder, P., and Baeuerle, P. A. (1991) EMBO J. 10, 2247–2258
23. Marui, N., Offermann, M. K., Swerlick, R., Kunisch, C., Rosen, A. C., Ahmad, M., Alexander, R. W., and Medford, R. M. (1993) J. Clin. Invest. 92, 1866–1874
24. Rapp, R. P., and Mossman, K. T. (1992) J. Cell Biol. 119, 131–138
25. Green, A. L., and Tischler, A. S. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 2424–2428
26. Lazzaroni, M. A., Cole, S., Cooke, C. A., Nelson, W. G., and Earnshaw, W. C. (1993) J. Cell Biol. 123, 7–22
27. Gavriliu, I., Sherman, Y., and Ben-Sasson, S. A. (1992) J. Cell Biol. 119, 493–501
28. Buttke, T. M., McCubrey, J. A., and Owen, T. C. (1993) J. Immunol. Methods 157, 233–240
29. Tsujimoto, Y. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 1958–1962
30. Meikrantz, W., Gisselbrecht, S., Tam, S. W., and Schlegel, R. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 7754–7758
31. Schaller, M., and Bossi, G. (1995) J. Cell Biol. 129, 465–471
32. Samuni, A., Aronovitch, J., Godinger, D., Chevion, M., and Czapski, G. (1983) Eur. J. Biochem. 137, 119–124
33. Rekten, K. L., Freeman, M. W., and Frei, B. (1993) J. Biol. Chem. 268, 1304–1309
34. Nelson, K. S., Bose, S. K., and McCord, J. M. (1994) Free Radical Biol. Med. 16, 195–200
35. Omar, B. A., and McCord, J. M. (1980) Free Radical Biol. & Med. 9, 473–478
36. Omar, B. A., Gadsden, R. M., Jordan, C. M., Striplin, S. P., Russel, W. J., Downey, J. M., and McCord, J. M. (1990) Free Radical Biol. & Med. 9, 465–471
37. Steinman, H. M. (1995) J. Cell Biol. 270, 3487–3490
38. Shimizu, Y., Eguchi, Y., Kosaka, H., Kamikawa, M., Matsuda, H., and Tsujimoto, Y. (1995) Nature 374, 811–813
39. Jacobson, M. D., and Raff, M. C. (1995) Nature 374, 814–816
40. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
Induction of Apoptosis by Pyrrolidinedithiocarbamate and N-Acetylcysteine in Vascular Smooth Muscle Cells
Jer-Chia Tsai, Mukesh Jain, Chung-Ming Hsieh, Wen-Sen Lee, Masao Yoshizumi, Cam Patterson, Mark A. Perrella, Carol Cooke, Hong Wang, Edgar Haber, Robert Schlegel and Mu-En Lee

J. Biol. Chem. 1996, 271:3667-3670.
doi: 10.1074/jbc.271.7.3667

Access the most updated version of this article at http://www.jbc.org/content/271/7/3667

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 49 references, 20 of which can be accessed free at http://www.jbc.org/content/271/7/3667.full.html#ref-list-1