Endothelial Cell Apoptosis Induced by Oxidized LDL Is Associated with the Down-regulation of the Cellular Caspase Inhibitor FLIP*

(Received for publication, September 16, 1998)
Masataka Sata‡ and Kenneth Walsh¶§

From the 3Division of Cardiovascular Research, St. Elizabeth’s Medical Center, Tufts University School of Medicine, Boston, Massachusetts 02135 and the 3Program in Cell, Molecular, and Developmental Biology, Sackler School of Biomedical Sciences, Tufts University, Boston, Massachusetts 02111

Fas (CD-95/APO-1) is a death receptor that initiates an apoptotic signal when activated by its ligand, FasL. Normal vascular endothelial cells are resistant to Fas-mediated apoptosis though they express both Fas and FasL. Oxidized low density lipoprotein (OxLDL) or lysophosphatidylcholine (LPC), a major component of OxLDL, induces endothelial cell suicide by sensitizing endothelial cells to Fas-mediated apoptosis. Here, we show that endothelial cell apoptosis by OxLDL and LPC-C16:0 was dose-dependent and correlated with down-regulation of FLICE-inhibitory protein (FLIP), an intracellular caspase inhibitor. FLIP down-regulation also occurred when endothelial cells were treated with toxic doses of LPC-C18:0 or minimally modified low density lipoprotein (LDL). In contrast, FLIP was not down-regulated by native LDL, acetylated LDL, LPC-C12:0, cholesterol, or 7-ketocholesterol, which are not toxic to endothelial cells. The cytotoxicity of oxidized lipids was reversed by transfecting endothelial cells with a FLIP expression plasmid. The results demonstrate, for the first time, FLIP regulation under conditions that lead to pathological tissue destruction.

---

Fas (CD95/Apo-1) is a type I transmembrane protein belonging to the tumor necrosis factor receptor family (1). Fas triggers an apoptosis-inducing signal when activated by its ligand, Fas ligand (FasL).1 FasL can induce apoptosis in Fas-bearing cells under normal conditions (5, 28). Recently, we documented that endothelial cells expressing both Fas and FasL do not undergo suicide or fratricide under normal conditions (4, 5), but these cells can become dramatically sensitized to the Fas-mediated apoptosis in response to specific stimuli or injuries (6–9). The increased sensitization to Fas/FasL-mediated suicide may play a role in regulation of physiological T cell number (4) and in pathological tissue destruction (10). However, the mechanisms by which stimuli sensitize cells to Fas-mediated apoptosis remains to be elucidated.

Caspases are cysteine proteases that play a central role in Fas-mediated apoptosis signaling pathway (11). Upon ligand activation, Fas is oligomerized (3) and recruits Fas-associated death domain protein (FADD) and pro-FLICE (FADD-homologous ICE-like protease), resulting in proteolytic activation of FLICE (12, 13). Active FLICE is released into the cytosol and triggers a cascade of caspases (11). FLIP (for FLICE-inhibitory protein) is a recently identified FADD-binding suppressor of apoptosis (14–16). Multiple isoforms are predicted based upon the analysis of FLIP cDNAs (14–16), two of which designated FLIPl (long FLIP isoform) and FLIps (short FLIP isoform) have been isolated from activated human peripheral blood leukocytes (14). All isoforms of FLIP contain FADD binding domain but lack the active-center cysteine residue and may function as dominant negatives for FLICE, thus blocking Fas-mediated apoptosis (14–16). FLIP isoforms are expressed during the early stage of T-cell activation and disappear when T cells become susceptible to Fas ligand-mediated apoptosis (14, 17), suggesting that the levels of intracellular FLIP may determine sensitivity of the cells to Fas-mediated apoptosis. FLIP is reported to inhibit Fas-mediated apoptosis (14, 18) and an apoptosis-protective role has been found in the viral FLICE-inhibitory proteins (19, 20). However, the role of FLIP is controversial as in some cell types its overexpression can induce apoptosis (14, 15, 21, 22).

It is firmly established that elevated levels of oxidized low density lipoprotein (LDL) are associated with accelerated atherogenesis (23, 24). OxLDL and its lipid constituents have numerous detrimental effects on endothelial cell function, including the induction of apoptosis (25–27). Vascular endothelial cells express functional FasL and detectable Fas on their cell surface, but they are resistant to Fas-mediated apoptosis under normal conditions (5, 28). Recently, we documented that OxLDL induces endothelial cell suicide through Fas-FasL interaction by sensitizing endothelial cells to Fas-mediated apoptosis (29). Here, we show that FLIP may play a role in regulating the sensitivity of endothelial cells to Fas-mediated apoptosis. This is the first report of FLIP regulation outside of the immune system and under conditions of pathological tissue destruction.

EXPERIMENTAL PROCEDURES

Cell Viability Assay and Reagents—Human umbilical vein endothelial cells (HUVECs) were isolated as described (30) and cultured in endothelial growth medium (EGM; Clonetics, San Diego, CA). HUVECs cultured in a 96-well plate at 80% confluency were incubated with OxLDL or LPC at indicated doses for 16 h. Cell viability was measured by means of MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (31). LDL was isolated by sequential ultracentrifugation (d = 1.019–1.063) from freshly drawn, citrated normalploidemic human plasma to which EDTA was added (32). LDL was oxidized in the presence of 5 μM CuSO4 for 24 h at 25 °C, and the degree of oxidation was assessed by the increase of mobility on 1% agarose gel (1.4 versus native LDL) (32). Minimally modified LDL (MM-LDL) was prepared by expressing both Fas and FasL do not undergo suicide or fratricide.
dialyzing native LDL against 9 μM FeSO₄ in phosphate-buffered saline for 72 h at 4 °C as described (33). The electrophoretic mobility increased 1.1 to 1.2 versus native LDL. Acetylation of LDL was performed with excess acetic anhydride. All lipid reagents were purchased from Sigma.

**Anti-FLIP Antibody**—An antiserum against human FLIP was generated in New Zealand White rabbits against a peptide spanning amino acids 2–27 (SAEVHQVEALDTDEKEMLLFLCRD) synthesized using the multiple antigen technology (34). The IgG fraction of the antiserum was isolated using E-Z-SEP kit (Amersham Pharmacia Biotech, Piscataway, NJ) and affinity-purified on the corresponding peptides coupled to Affi-Gel 15 gel (Bio-Rad, Hercules, CA).

**Immunoblot Analysis**—HUVECs were treated with OxLDL (0, 100, 200, 300, and 400 μg of protein/ml) or LPC (0, 20, 40, 60, 80, and 100 μM) for 21 h at 37 °C. The cells were lysed with a lysis buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor mixture (Sigma) in phosphate-buffered saline. The protein content was measured with BCA protein assay reagent (Pierce, Rockford, IL). The cell lysates (15 μg for each lane) were electrophoresed through a 10% SDS-polyacrylamide gel and electrotransferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The membrane was incubated with either anti-FLIP, anti-α-tubulin (Calbiochem, San Diego, CA), anti-Bcl-2 (Transduction Laboratories), anti-Bcl-x (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-Bax (Santa Cruz Biotechnology) antibody. Antibody binding was detected using horseradish peroxidase-conjugated sheep antibody to mouse Ig or rabbit Ig (Amersham Pharmacia Biotech) and enhanced chemiluminescence system (Amersham Pharmacia Biotech).

**Transfection of HUVECs with FLIP Expression Plasmid**—HUVECs cultured in 24-well plates were transfected with β-galactosidase expression plasmid (1.5 μg) and a pcR3-based (Invitrogen, Carlsbad, CA) expression plasmid (0.15 μg) for human long form of FLIP (FLIP₁), a generous gift from Dr. J. Tschopp), Bcl-2, or Bcl-X₁ using the Superfect procedure (Qiagen). After 48 h, HUVECs were incubated with 60 μM LPC-C16:0 for 24 h, fixed in 2% formaldehyde and 0.2% glutaraldehyde for 10 min, and stained with X-gal (Amersham Pharmacia Biotech). The number of viable blue cells attaching to the culture plate was counted as described (35–37).

**RESULTS AND DISCUSSION**

To study FLIP regulation under conditions of endothelial cell death, we initially determined the parameters influencing oxidized lipid-induced apoptosis. HUVECs were treated with different doses of OxLDL or LPC-C16:0 for 24 h, and mitochondrial function was assessed by the MTT assay, an indicator of cell viability (31, 38). Consistent with previous reports of cytotoxicity by these agents (26, 39), OxLDL at or above 300 μg of protein/ml (Fig. 1A) and LPC-C16:0 at or above 60 μM (Fig. 1B) markedly decreased mitochondrial function. Lower doses of OxLDL and LPC-C16:0 were slightly stimulatory, consistent with reports that these agents can stimulate growth (40, 41).

To study regulation of FLIP under conditions of OxLDL- or LPC-induced endothelial cell apoptosis, a polyclonal antibody was generated against the 26-amino acid sequence of the N-terminus of FLIP. This sequence is common among all of the reported isoforms (14–16). Immunoblotting revealed that untreated HUVECs express a 55-kDa immunoreactive protein that has an identical mobility to the positive control, an extract from COS cells transfected with FLIP₁ expression plasmids. Expression levels of the previously reported FLIP₁ were very low or undetectable in HUVECs. The Bcl-2 family of proteins functions as positive and negative regulators of apoptosis (42), including Fas-mediated cell death (43). In contrast to FLIP, OxLDL treatment had no effect on the expression of the pro-apoptotic protein Bax or the anti-apoptotic proteins Bcl-x or Bcl-2 (Fig. 2C). Furthermore, LPC did not affect the expression of genes encoding the death-
signaling molecules, FADD, FAP, FAF, DR3, TRAIL, TNFR-p55, or RIP (not shown). These data suggest that oxidized lipids specifically modulate FLIP levels, and that they do not have widespread effects on the expression of other apoptosis-regulatory molecules.

Other lipid species were examined for their ability to induce cytotoxicity and down-regulate FLIP. Native LDL did not influence HUVEC viability (Fig. 3A). Acetylated LDL, which is internalized by endothelial cells but lacks biological effects (44, 45), also did not affect endothelial cell viability. On the other hand, MM-LDL, which promotes atherogenesis by acting as an inflammatory agent (33, 46), induced endothelial cell death. LPC-C12:0, which does not inhibit endothelial cell migration (47), also did not affect endothelial cell viability, whereas LPC-C18:0, a component of OxLDL, induced endothelial cell death. Finally, cholesterol and 7-ketocholesterol did not kill endothelial cells. Immunoblotting against FLIP revealed that cytotoxic concentrations of MM-LDL and LPC-C18:0 down-regulated FLIP expression level, whereas nontoxic lipids did not (Fig. 3B). These results further suggest that FLIP down-regulation accounts for endothelial cell sensitization to Fas-mediated apoptosis.

To examine the functional significance of FLIP down-regulation in sensitization of human endothelial cells to Fas-mediated apoptosis, we tested whether ectopic expression of FLIP prevented endothelial cells from LPC-induced apoptosis. Consistent with observations that Bcl-XL but not Bcl-2 protects Fas-mediated apoptosis (43), ectopic expression of Bcl-XL also protected LPC-induced endothelial cell apoptosis, whereas Bcl-2 did not.

Vascular endothelial cells express both Fas and FasL, but they are normally resistant to Fas-mediated apoptosis (5, 28). Oxidized lipids sensitize endothelial cells to Fas-mediated apoptosis leading to cell suicide (29). Here, we found that oxidized lipids down-regulate FLIP protein. FLIP down-regulation correlates with a loss of endothelial cell viability, and oxidized lipid-induced cell death was reversed by forced FLIP expression. Previously, FLIP regulation has only been described during T cell activation where it may control Fas-mediated suicide (14, 17). The findings described herein suggest that FLIP levels may play a role in determining endothelial cell sensitivity to oxidized lipid-induced apoptosis, and they indicate that FLIP may be a target of agents that cause pathological cell death.

Acknowledgment—We thank Dr. J. Tschopp for providing the FLIP plasmids.

REFERENCES

1. Nagata, S., and Golstein, P. (1995) Science 267, 1449–1456
2. Hahne, M., Rinaldi, D., Schrieter, M., Romero, P., Schreier, M., French, L. E., Schneider, P., Bornand, T., Fontana, A., Liendr, D., Cerottini, J.-C., and Tschopp, J. (1996) Science 274, 1363–1366
3. Nagata, S. (1997) Cell 88, 355–365
4. Klas, C., Debatin, K.-M., Jonker, B. R., and Krammer, P. H. (1993) Int. Immunol. 5, 625–630
5. Sato, M., and Walsh, K. (1998) Nat. Med. 4, 415–420
6. Hueber, A.-O., Zornig, M., Lyon, D., Suda, T., Nagata, S., and Evans, G. I. (1997) Science 278, 1305–1309
7. Reap, E. A., Roof, K., Maynor, K., Barrero, M., Bookier, J., and Cohen, P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5750–5755
8. Muller, M., Strand, S., Hug, H., Heinemann, E.-M., Walczak, H., Hofmann, W. J., Stremmel, W., Krammer, P. H., and Galle, P. R. (1997) J. Clin. Invest. 99, 463–473
9. Friesen, C., Herr, I., Krammer, H., and Debatin, K.-M. (1996) Nat. Med. 2, 574–577
10. Strand, S., Hofmann, W. J., Grambihler, A., Hug, H., Volkman, M., Otto, G., Wesch, H., Marani, S. M., Hack, V., Stremmel, W., Krammer, P. H., and Galle, P. R. (1998) Nat. Med. 4, 588–593
11. Salvesen, G. S., and Dixit, V. M. (1997) Cell 91, 443–446
12. Montecucco, J. P., Scaffidi, C., Kischkel, F. C., Shimizu, A., Marzo, M., Krammer, P. H., and Peter, M. E. (1997) EMBO J. 16, 2794–2804
13. Yang, X., Chang, H. Y., and Baltimore, D. (1998) Mol. Cell 1, 319–325
14. Imler, J. L., Fromm, M., Hahne, M., Schneider, M., Hofmann, K., Steiner, V., Bodmer, J.-L., Schrieter, M., Bursa, K., Mattmann, K., Rimpld, D., French, L., and Tschopp, J. (1997) Nature 388, 190–195
15. Ha, S., Vincenz, C., Ni, J., Gents, R., and Dixit, V. M. (1997) J. Biol. Chem. 272, 17269–17277
16. Srinivasula, S. M., Ahmad, M., Otiile, S., Bullrich, F., Banks, S., Fernandes-Alnemri, T., Croce, C. M., Litwack, G., Tomasselli, K. J., Armstrong, R. C., and Alnemri, E. S. (1997) J. Biol. Chem. 272, 13542–13545
17. Raffaelli, Y., Van Parijs, L., London, C. A., Tschopp, J., and Abbas, A. K. (1998) Immunity 8, 615–623
18. Hiti, H., Lorenz, J., Kitada, S. I., Fisher, J., LaBarge, M., Ring, H. Z., Francke, U., Reed, J. C., Kinoshita, S., and Nolan, G. P. (1998) Immunity 8, 461–471
19. Thome, M., Schneider, P., Hofmann, K., Fickenscher, H., Meinl, E., Neipel, F., Mattmann, C., Burns, K., Bodmer, J. L., Schroeter, M., Scaffidi, C.,
Krammer, P. H., Peter, M. E., and Tschopp, J. (1997) *Nature* **386**, 517–521
20. Hu, S., Vincenz, C., Buller, M., and Dixit, V. M. (1997) *J. Biol. Chem.* **272**, 9621–9624
21. Shu, H. B., Halpin, D. R., and Goeddel, D. V. (1997) *Impunity* **6**, 751–763
22. Han, D. K. M., Chaudhary, P. M., Wright, M. E., Friedman, C., Trask, B. J., Riedel, R. T., Baskin, D. G., Schwartz, S. M., and Hood, L. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 11333–11338
23. Ross, R. (1993) *Nature* **362**, 801–809
24. Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C., and Witztum, J. L. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 11333–11338
25. Ross, R. (1993) *Nature* **362**, 801–809
26. Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C., and Witztum, J. L. (1989) *N. Engl. J. Med.* **320**, 915–924
27. Dimmeler, S., Haendeler, J., Galle, J., and Zeiher, A. M. (1997) *Circulation* **95**, 1760–1763
28. Escargueil-Blanc, I., Meilhac, O., Pieraggi, M.-T., Arnal, J.-F., Salvayre, R., and Negre-Salvayre, A. (1997) *Arterioscler. Thromb. Vasc. Biol.* **17**, 331–339
29. Harada-Shiba, M., Kinoshita, M., Kamido, H., and Shimokado, K. (1998) *J. Biol. Chem.* **273**, 9681–9687
30. Han, D. K. M., Chaudhary, P. M., Wright, M. E., Friedman, C., Trask, B. J., Riedel, R. T., Baskin, D. G., Schwartz, S. M., and Hood, L. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 11333–11338
31. Tanaka, M., Suda, T., Haze, K., Nakamura, N., Sato, K., Kimura, F., Motoyoshi, K., Miruku, M., Tagawa, S., Ohga, S., Hatake, K., Drummond, A. H., and Nagata, S. (1996) *Nature* **386**, 517–521
32. Galle, J., Stunz, P., Schollmeyer, P., and Wanner, C. (1995) *Kidney Int.* **47**, 45–52
33. Liao, F., Berliner, J. A., Mehrabian, M., Navah, M., Demer, L., Lusis, A. J., and Fogelman, A. M. (1991) *J. Clin. Invest.* **87**, 2253–2257
34. Francis, M. J., Hastings, G. Z., Brown, F., McDermed, J., Lu, Y. A., and Tam, J. P. (1991) *Immunity* **3**, 249–254
35. Perlman, H., Sata, M., Le Roux, A., Sedlak, T., Branellec, D., and Walsh, K. (1998) *EMBO J.* **17**, 3576–3586
36. Miura, M., Zhu, H., Rotello, R., Hartwig, E., and Yuan, J. (1993) *Cell* **75**, 653–660
37. Chittenden, T., Flemington, C., Houghton, A. B., Ebb, R. G., Gallo, G. J., Elangovan, B., Chinnadurai, G., and Lutz, R. J. (1995) *EMBO J.* **14**, 5589–5596
38. Weng, G. H., and Goeddel, D. V. (1994) *J. Immunol.* **152**, 1751–1755
39. Juckett, M. B., Balla, J., Balla, G., Jessurun, J., Jacob, H. S., and Vercellotti, G. M. (1995) *Am. J. Pathol.* **147**, 782–789
40. Chen, J. K., Hoshi, H., McClure, D. B., and McKeehan, W. L. (1986) *J. Cell. Physiol.* **129**, 207–214
41. Sakai, M., Miyazaki, A., Hakamata, H., Sato, Y., Matsumura, T., Kobori, S., Shichiri, M., and Horiuchi, S. (1996) *Arterioscler. Thromb. Vasc. Biol.* **16**, 600–605
42. Reed, J. C. (1997) *Nature* **387**, 773–776
43. Perez, D., and White, E. (1998) *J. Cell Biol.* **141**, 1255–1266
44. Stein, O., and Stein, Y. (1989) *Biochim. Biophys. Acta* **620**, 631–635
45. Sugiyama, S., Kugiyama, K., Ogushi, M., Fujimoto, K., and Yasue, H. (1994) *Circ. Res.* **74**, 565–575
46. Berliner, J. A., Territo, M. C., Sevanian, A., Ramin, S., Kim, J. A., Bamshad, B., Esterson, M., and Fogelman, A. M. (1990) *J. Clin. Invest.* **85**, 1260–1266
47. Murugesan, G., and Fox, P. L. (1996) *J. Clin. Invest.* **97**, 2796–2744
Endothelial Cell Apoptosis Induced by Oxidized LDL Is Associated with the Down-regulation of the Cellular Caspase Inhibitor FLIP
Masataka Sata and Kenneth Walsh

J. Biol. Chem. 1998, 273:33103-33106.
doi: 10.1074/jbc.273.50.33103

Access the most updated version of this article at http://www.jbc.org/content/273/50/33103

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 47 references, 17 of which can be accessed free at http://www.jbc.org/content/273/50/33103.full.html#ref-list-1