Rev Esp Patol 1999; Vol. 32, N° 2: 143-147

Revisión

Role of apoptosis in atherogenesis

V. Lesauskaite

Kaunas Medical University, Institute of Cardiology, Sukileliu 17, Kaunas LT-3000, Lithuania.

The general rule for a cell is divide or die...
M.J. Davies (1)

This general rule cited by Davies also applies to the cells involved in atherogenesis. The most widely accepted etiology of atherogenesis is currently the "response-to-injury" hypothesis which postulates that the initiation of lesions involves proliferation of intimal smooth muscle cells (SMCs) (2). Only later does the death of the lipid-filled foam cells become of great importance during the formation of atherosclerotic plaques which contain a core of acellular lipid. The mechanism ultimately responsible for necrosis is far from clear, but popular explanations include a combination of hypoxia and cytotoxic lipid peroxides which form within the cell cytoplasm (1). Recent studies have shown that cellular death by apoptosis also takes place within atherosclerotic lesions (3-5).

APOPTOSIS IN ATHEROSCLEROTIC LESIONS

Increased apoptosis has been found in atherosclerotic lesions from both humans and animals and appears to mediate tissue turnover and lesion development (5, 6). The majority of apoptotic cells in atherosclerotic lesions were found to be modulated SMCs and macrophages (3, 4). Apoptotic cells were present in the intima in all stages of atherosclerosis. In the initial lesions, only a few cells appeared to be undergoing apoptosis. In contrast, in advanced lesions, many cells were found to be undergoing apoptosis (7). All investigators are in agreement that in the atherosclerotic lesions, apoptosis mainly affects macrophages and SMCs. However, various authors have presented significantly differing data concerning the rate of apoptosis in cells. The reported values for the number of apoptotic cells range from 2% (8, 9) up to 30% to 40% (3, 4). Such differences might be due to methodological problems in the detection of apoptosis; these differences will be further discussed below.

The significance of apoptosis of SMCs is different than for macrophages. Apoptotic SMCs were found to be localized mainly in the fibrotic portion of the atheroma, whereas apoptotic macrophages clustered near or within the lipid-rich core of the lesion (4). Loss of SMCs in the fibrous cap could have serious consequences since they are the only cells in cap that can synthesize collagen thereby maintaining its tensile strength. Thus, any

increased apoptosis of SMCs within this vulnerable region of the plaque could result in weakening and rupture of the fibrous cap (10, 11). Necrosis and apoptosis of macrophages probably promotes the formation and enlargement of the lipid core (12), whose size correlates with plaque instability (13). On the other hand, Kockx and Herman (11) suggest that increased macrophage apoptosis can lead to plaque stabilization through decreased breakdown of collagen.

Recent studies have shown that calcification is an important consequence of apoptosis (14). If this is correct, then calcification of atherosclerotic plaques might also be initiated by apoptotic cell death via the formation of matrix vesicles. These matrix vesicles are cytoplasmic remnants of apoptotic smooth muscle cells and are known to initiate plaque calcification (11, 15).

Some studies (8, 16) suggest that apoptosis may modulate the cellularity of lesions that produce human vascular obstruction. This is particularly true for those lesions with greater evidence of extensive proliferative activity as in the formation of a neointima or in a restenosis. Bauriedel *et al.* (16) showed that restenotic lesions contained fewer apoptotic cells than primary atherosclerotic lesions, while there were no differences in cellular necrosis. As such, a threshold level of apoptosis may be an important mechanism leading to the development of restenotic intimal lesions after surgical procedures. Apoptosis may also regulate cell mass in the normal arterial wall. The higher rates of apoptosis seen in SMCs may ultimately contribute to plaque rupture and breakdown and, as a result, to the clinical manifestation of atherosclerosis (10). On the other hand, it was concluded that apoptotic vascular SMCs possess a significant ability to generate thrombin secondary to their exposure to phosphatidylserine. Apoptotic cells within atherosclerotic plaques may thus facilitate local activation of thrombin, thereby contributing to disease progression (17). As apoptosis may weaken the atherosclerotic plaque, predispose it to rupture, and lead to the formation of a thrombus, it would seem that an investigation of the mechanisms regulating apoptosis would be of great clinical significance.

REGULATION OF APOPTOSIS IN ATHEROSCLEROSIS

In general, the regulation of apoptosis occurs in two distinct phases. Firstly, there is integration of the many pro- and antiapoptotic signals arising from the cell surface, which are transmitted by specific second messenger pathways. Anthropomorphically speaking, this constitutes the "decision" phase and includes the expression of specific pro- and antiapoptotic gene products, such as proto-oncogenes, tumor suppressor genes, etc. If the summation of these signals leads to an induction of apoptosis, there is an "execution" phase. During this phase an irreversible activation of a cascade of cysteine proteases occurs. This culminates in actual cell disintegration. In addition, the "decision" phase can be bypassed by direct activation of cysteine proteases (18).

Bennet *et al.* (10) showed that the proto-oncogene Bcl-2 is able to protect plaque-derived SMCs against apoptosis *in vitro*. The same researchers identified *c-myc* and *p53*, genes important for controlling cell proliferation, as also regulating apoptosis in smooth muscle. They showed that vascular SMCs from human plaques have increased sensitivity to tumor suppressor gene *p53*-mediated apoptosis compared with normal vascular SMCs (19). Kockx *et al.* (20) demonstrated that SMCs in the deep layer of human fatty streaks express Bax, a proapoptodtic protein of the Bcl-2 family, which increases the susceptibility of these cells to apoptosis. These findings might be important to our understanding of the transition of fatty streaks into atherosclerotic plaques, which are characterized by regions of cell death.

It was revealed that H_2O_2 and its derivative, ·OH, might have a causal relationship to apoptosis of vascular SMCs in atherosclerosis and restenosis (21). This study showed that the death of foam cells at the margin of the lipid core results from both necrosis and apoptosis, and that remnants of the apoptotic nuclei are present within the lipid core. The cause of foam cell death in atherogenesis is unknown, but oxidized low density lipoprotein (ox-LDL) can cause macrophage apoptosis *in vitro*. This might play a role in the formation and enlargement of the lipid core (12). It was concluded that ox-LDL induces apoptosis in SMCs as well (22). It seems that ultraviolet light-induced apoptosis of arterial SMCs and macrophages (23) is also due to strongly oxidized LDL (24). Nitric oxide (NO) can also induce apoptosis in SMCs. Therefore, it is suggested that NO acts as a mediator in the development of atherosclerotic lesions via alterations in the number of SMCs (25). This suggests that apoptosis caused by free radicals or by NO is induced by *p53* up-regulation (18, 26).

Another mechanism of regulating apoptotic cell death is by specific cell death receptors. The best studied of these receptors is Fas. It was shown that Fas/apolipoprotein-I (APO-I) is present in foam cells. These data suggest that Fas/APO-I-regulated apoptosis is involved in the development of advanced human atherosclerotic lesions, and that this probably determines the amount of tissue mass in the diseased vessel (10). Data suggest that activation of the Fas cell death-signaling pathway by cytokines derived from activated macrophages and T-cells contributes to the induction of smooth muscle apoptosis during atherogenesis (18). It was shown (27) that in culture, stimulation with interferon-γ, tumor necrosis factor-α (TNF-α), and interleukin-1β increased expression of Fas in SMCs. Later incubation with anti-Fas antibody triggered apoptosis of the cytokine- primed SMCs. This route provides a mechanism as to how immune cells and their cytokines promote this cell death process in a manner related to vascular remodelling and plaque rupture.

Calcium channel blockers (verapamil) not only inhibit vascular smooth muscle proliferation, but can also induce their apoptosis (28). The ability of cyclosporin A treatments to reduce transplant atherosclerosis is attributed to its ability to inhibit ox-LDL-, TNF-α- or angiotensin II-induced apoptosis of endothelial cells (29). In the future, a fuller understanding of the regulation of apoptosis may help to guide our efforts in atherogenesis management.

METHODOLOGICAL PROBLEMS IN RECOGNIZING APOPTOTIC CELLS IN ATHEROSCLEROTIC LESIONS

The essential process in apoptosis is the production of endonucleases which begin to cut the DNA between nucleosomes into short 180 base pair fragments. The detection of this DNA fragmentation is thus a marker of apoptotic cell death (30). Various methods are used to detect apoptosis. The morphological features of apoptosis are easily recognized ultrastructurally. The cells undergoing apoptosis shrink and become denser, their chromatin becomes pyknotic and applied to the nuclear membrane, their nuclei may also disintegrate, and the cell ultimately emits processes that often contain pyknotic nuclear fragments (31). A complicating factor of morphological detection methods is that apoptotic cells are usually observed with low frequency due to the short duration of the changes (32).

A more sensitive method for detecting apoptosis is by the electrophoretic separation of the fragmented DNA in a gel. The DNA fragments can be recognized by a characteristic ladder-like pattern with multiple bands at short regular intervals. A limitation to this method is that it gives no indication as to which cell type is undergoing apoptosis. Thus, the method is useful when the cells undergoing apoptosis are uniform, but not for an investigation involving a more multivariate system as in atherosclerotic plaques.

Two enzymatic reactions are also used for *in situ* detection of apoptotic cell death. One is based on *in situ* end-labeling (ISEL) of fragments in the DNA strand through the incorporation of biotinylated nucleotides using DNA polymerase I and their subsequent visualization with diaminobenzidine via peroxidase-conjugated avidin (32). DNA polymerase I adds nucleotides to the 3'-hydroxyl ends of DNA strands in the presence of a template, extending the strand in the 5' to 3' direction (33). Thus, incorporation of nucleotides can occur when one strand of a double-stranded DNA molecule is nicked. A second method of assessing apoptosis involves terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine 5-triphosphate (dUTP)-biotin nick end-labeling (TUNEL) (34). In this method, dUTP-biotin is transferred by TdT to the free 3' end of cleaved DNA. This technique is of particular interest as it labels the blunt ends of only double-stranded DNA fragments. Such double-strand fragments typically occur only during apoptotic DNA fragmentation. Gold *et al.* (35) showed that TUNEL labeling was more sensitive as compared to ISEL. This can be explained by the presumption that double stranded DNA fragments occur at a much higher frequency than single strand DNA fragment in apoptotic cells. Thus, DNA polymerase I works mainly by its exonucleolytic activity to sufficiently amplify the signal at the site of a rare single strand DNA fragment in apoptotic cells (35).

For the detection of apoptosis in atherosclerotic lesions, the enzymatic ISEL or TUNEL reactions are the most commonly used. While the number of papers dealing with apoptosis in atherosclerosis increases, there has been a concomitant general increase in the number of papers evaluating methodological problems related to apoptosis. These methodological problems can lead to false-positive or false-negative reactions for apoptosis.

Thus, the interpretation of data obtained by *in situ* DNA end-labeling techniques for atherosclerotic plaques or for other pathological processes must be carried out with caution. Kockx *et al.* (15), for example, reported that the ISEL technique can also label non-nuclear structures in atherosclerotic plaques by nonspecific binding of the nucleotides to small calcium-containing vesicles within the plaques. This technical problem can be avoided by pretreating the tissue sections with such calcium chelating agents as EDTA or citrate (15).

Other factors that might result in TUNEL labeling must also be considered. The problem is that *in situ* DNA end-labeling is not specific for apoptosis. These techniques only show DNA strand fragments, which also occur during necrosis or autolytic cell death. According to Grasl-Kraup *et al.* (36) DNA fragmentation is common in different kinds of cell death. Therefore, its detection by *in situ* methods should not be considered a specific marker of apoptosis. Gold *et al.* (35) studied to what extent the use of ISEL and TUNEL labeling allowed one to differentiate between internucleosomal DNA degradation (typical for apoptosis), or more random DNA destruction (common in necrosis). They showed that the early stages of necrosis *in vitro* and *in vivo* were preferentially detected by staining with ISEL. TUNEL labeling, by contrast, was slightly more sensitive for the detection of apoptosis (35). Mundle and Raza (37) failed to confirm the findings of Gold *et al.* In their experience, the two methods detected apoptosis or necrosis with equal efficiency, but were more sensitive for apoptosis than for necrosis.

In addition to necrosis, other phenomena can also interfere with the TUNEL technique. Kockx *et al.* (38) demonstrated that besides apoptotic nuclei, nonapoptotic nuclei showing signs of active gene transcription are labeled by the TUNEL technique within an atherosclerotic plaque. They suggested that this is a consequence of the important conformational changes that occur in DNA structure during transcription.

The TUNEL technique is very sensitive and therefore needs a careful titration of TdT concentration. Hegyi *et al.* (39) found a high percentage (>40%) of TUNEL-stained nuclei in atherosclerotic plaques when the incubation with TdT working solution was carried out for 1 hour (37 °C) and TdT was applied at a concentration of 0.3 U/μl or higher. Decreasing the TdT incubation period to 30 min and reducing its concentration resulted in a decrease in the proportion of apoptotic cells by 2%. Thus, a failure to recognize the importance of critical adjustments specific to these techniques can produce exaggerated estimates of the number of apoptotic cells in tissue (39).

False-negative results for apoptosis can be obtained due to long formalin fixation (40). Increasing the fixation time reduces the sensitivity of ISEL and TUNEL for the detection of DNA fragmentation in paraffin sections. The sensitivity of both methods can be increased if the sections are pretreated with proteinase K before performing ISEL or TUNEL techniques (35).

The causes of over- or underestimating apoptotic cells in the interpretation of ISEL or TUNEL data obtained for atherosclerotic plaques and other pathological processes are of considerable interest. These data will not be truly meaningful and quantitative unless these methods are carefully standardized from laboratory to laboratory. Therefore, as Kokcx *et al.* (38) advise, one should always combine *in situ* labeling with additional techniques, such as the inclusion of markers of transcription or the use of morphological criteria.

REFERENCES

1. Davies MJ. *Apoptosis in cardiovascular disease.* Heart 1997; 77: 498-501.
2. Ross R, Glomset JA. *The pathogenesis of atherosclerosis I, II.* N Engl J Med 1976; 295: 369-377, 420-425.
3. Han DK, Haudenschild CC, Hong MK et al. *Evidence for apoptosis in human atherogenesis and in a rat vascular injury model.* Am J Pathol 1995; 147(2): 267-277.
4. Geng YJ, Libby P. *Evidence for apoptosis in advanced human atheroma. Colocalization with interleukin-1 beta-converting enzyme.* Am J Pathol 1995; 147(2): 251-256.
5. Geng YJ. *Regulation of programmed cell death or apoptosis in atherosclerosis.* Heart Vessels 1997; Suppl. 12: 76-80.
6. Harada K, Chen Z, Ishibashi S et al. *Apoptotic cell death in atherosclerotic plaques of hyperlipidemic knockout mice.* Atherosclerosis 1997; 135(2): 235-239.
7. Cai W, Devaux B, Schaper W, Schaper J. *The role of Fas/APO I and apoptosis in the development of human atherosclerotic lesions.* Atherosclerosis 1997; 131(2): 177-186.
8. Isner JM, Kearny M, Bortman S, Passeri J. *Apoptosis in human atherosclerosis and restenosis.* Circulation 1995; 91(11): 2703-2711.
9. Kockx MM, De-Meyer GR, Muhring J et al. *Distribution of cell replication and apoptosis in atherosclerotic plaques of cholesterol-fed rabbits.* Atherosclerosis 1996; 120(1-2): 115-124.
10. Bennett MR, Evan GI, Schwartz. *Apoptosis of human vascular SMCs derived from normal vessels and coronary atherosclerotic plaques.* J Clin Invest 1995; 95(5): 2266-2274.

11. Kockx N4M, Herman AG. *Apoptosis in atherogenesis: Implications for plaque destabilization.* Eur Heart J 1998; 19(Suppl. G): G23-28.
12. Hegyi L, Skepper JN, Cary NR, Mitchinson MJ. *Foam cell apoptosis and the development of the lipid core of human atherosclerosis.* J Pathol 1996; 180(4): 423-429.
13. Libby P, Geng YJ, Aikawa M et al. *Macrophages and atherosclerotic plaque stability.* Curr Opin Lipidol 1996; 7(5): 330-335.
14. Kim KM. *Apoptosis and calcification.* Scanning Microsc 1995; 9(4): 1137-1175.
15. Kockx MM, Muhring J, Bortier H et al. *Biotin- or digoxigenin-conjugated nucleotides bind to matrix vesicles in atherosclerotic plaques.* Am J Pathol 1996; 148(6): 1771-1777.
16. Bauriedel G, Schluckebier S, Hutter R et al. *Apoptosis in restenosis versus stable-angina atherosclerosis.* Arterioscler Thromb Vasc Biol 1998; 18(7): 1132-1139.
17. Flynn PD, Byme CD, Baglin TP et al. *Thrombin generation by apoptotic vascular SMCS.* Blood 1997; 89(12): 4378-4384.
18. Bennett MP, Boyle JJ. *Apoptosis of vascular smooth muscle cells in atherosclerosis.* Atherosclerosis 1998; 138: 3-9.
19. Bennett MP, Littlewood TD, Schwartz SK, Weissberg PL. *Increased sensitivity of human vascular SMCs from atherosclerotic plaque to p53-mediated apoptosis.* Circ Res 1997; 81(4): 591-599.
20. Kockx MM, De Meyer GR, Muhring J et al. *Apoptosis and related proteins in different stages of human atherosclerotic plaques.* Circulation 1998; 97(23): 2307-2315.
21. Li PF, Dietz R, von Harsdorf R. *Reactive oxygen species induce apoptosis of vascular SMC.* FEBS Lett 1997; 404(2-3): 249-252.
22. Nishio E, Arimura S, Watanabe Y. *Oxidized LDL induces apoptosis in cultured SMCS: A possible role for 7-ketocholesterol.* Biochem Biophys Res Commun 1996; 223(2): 413-418.
23. López-Candales A, Hohnes DR, Scott MJ et al. *Effects of ultraviolet light in vascular cells in vitro and in intact atherosclerotic explants: Potential role of apoptosis in vascular biology.* Biochem Cell Biol 1996; 74(3): 333-345.
24. Bjorkerud B, Bjorkerud S. *Contrary effects of lightly and strongly oxidized LDL with potent promotion of growth versus apoptosis on arterial SMCS, macrophages, and fibroblasts.* Arterioscler Thromb Vasc Biol 1996; 16(3): 416-424.
25. Nishio E, Fukushirna K, Shiozaki M, Watanabe Y. *Nitric oxide donor SNAP induces apoptosis in SMCs trough cGNT-independent mechanism.* Biochem Biophys Res Commun 1996; 221(1): 163-168.
26. Schwartz SM, Bennett NM. *Death by any other name.* Am J Pathol 1995; 147(2): 229-234.
27. Geng YJ, Henderson LE, Levesque EB et al. *Fas is expressed in human atherosclerotic intima and promotes apoptosis of cytokine-primed human vascular SMCS.* Arterioscler Thromb Vasc Biol 1997; 17(10): 2200-2208.
28. Leszczynski D, Zhao Y, Luokkamaki M, Foegh ML. *Apoptosis of vascular SMCS. Protein Kinase C and oncoprotein Bcl-2 are involved in regulation of apoptosis in nontransformed rat vascular SMCS.* Am J Pathol 1994; 145(6): 1265-1270.
29. Walter DH, Haendeler J, Galle J et al. *Cyclosporin A inhibits apoptosis of human endothelial cells by preventing release of cytochrome C from mitochondria.* Circulation 1998; 98(12): 1153-1157.
30. Arends MJ, Morris RG, Wyllie AH. *Apoptosis: The role of the endonuclease.* Am J Pathol 1990; 136: 593-608.
31. Majno G, Joris I. *Apoptosis, oncosis, and necrosis: An overview of cell death.* Am J Pathol 1995; 146(1): 3-15.
32. Wijasman JH, Jonker RR, Keijzer R et al. *A new method to detect apoptosis in paraffin sections: In situ end-labeling of fragmented DNA.* J Histochem Cytochem 1993; 41(1): 712.
33. Lehninger AL. *Biochemistry.* 2nd ed. Worth Publishers, New York 1975.
34. Yasuda M, Umemura S, Osamura RY et al. *Apoptotic cells in the human endometrium and placental villi: Pitfalls in applying the TUNEL method.* Arch Histol Cytol 1995; 58: 185-190.
35. Gold R, Schmied M, Giegerich G et al. *Differentiation between cellular apoptosis and necrosis by the combined use of in situ tailing and nick translation techniques.* Lab Invest 1994; 71(2): 219-225.
36. Grasl-Kraupp B, Ruttkay-Nedecky B, Koudelka H et al. *In situ detection of fragmented DNA (TUNEL Assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: Cautionary note.* Hepatology 1995; 21(5): 1465-1468.
37. Mundle SD, Raza A. *Correspondence. The two in situ techniques do not differentiate between apoptosis and necrosis but rather distinct patterns of DNA fragmentation in apoptosis.* Lab Invest 1995; 72(5): 611-612.
38. Kockx MM, Muhring J, Knaapen MWM, de Meyer GRY. *RNA synthesis and splicing interferes with DNA in situ end labeling techniques used to detect apoptosis.* Am J Pathol 1998; 152(4): 885-888.
39. Hegyi L, Hardwick SJ, Mitchinson MJ. *Correspondence.* Am J Pathol 1997; 150(1): 371.
40. Davison FD, Groves M, Scaravilli F. *The effects of formalin fixation on the detection of apoptosis in human brain by in situ end-labeling of DNA.* Histochem J 1995; 27(12): 983-988.