Brief Reviews

Apoptosis in the Atherosclerotic Plaque
Quantitative and Qualitative Aspects

Mark M. Kockx

Abstract—Several laboratories have demonstrated the presence of apoptotic cell death in atherosclerotic plaques. Apoptosis occurs in at least 2 stages. The final "execution" phase, which includes DNA fragmentation, is brief (~6 hours) and irreversible and can be detected by the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) technique. The TUNEL technique is only selective (rather than specific) for apoptotic nuclei, because these contain a far greater degree of DNA fragmentation than do nonapoptotic nuclei. Nonapoptotic cell nuclei that show high levels of RNA synthesis and splicing can also be labeled. This could explain the large variation in the reported percentages of TUNEL-positive nuclei in the plaques. Therefore, the TUNEL technique should be combined with additional techniques, such as markers of transcription and morphological criteria. Recent studies indicate that human fatty streaks differ from adaptive intimal thickenings by the presence of cells containing pro-apoptotic proteins. However, apoptotic cell death is present only in advanced atherosclerotic plaques that show a dense macrophage infiltration. This indicates that although both smooth muscle cells and macrophages within the human fatty streaks become susceptible to apoptosis, additional factors (mainly macrophage- and lipid-derived factors) are necessary to terminate the cell death pathway. (Arterioscler Thromb Vasc Biol. 1998;18:1519-1522.)

Key Words: atherosclerosis ■ apoptosis ■ BAX ■ TUNEL ■ lipids

Apoptosis: Definition, Detection Methods, and Their Possible Pitfalls

Kerr et al have introduced the term “apoptosis” to distinguish a special form of cell death different from necrosis. Apoptotic cell death is characterized by a series of morphological changes detectable by light and electron microscopy, starting from the shrinkage of the cell membrane, to condensation of nuclear chromatin, cellular fragmentation, and finally the engulfment of the apoptotic bodies by neighboring cells.

Although the term apoptosis was introduced only 25 years ago, typically apoptotic morphology was described by embryologists at the beginning of this century. Embryologists recognized apoptosis as a mechanism to counterbalance the excess cellular proliferation during the development of organs and limbs. More recently, apoptosis has also been implicated in the development of arteries. Cho et al have studied apoptosis during lamb vessel development, and Slomp et al focused on apoptosis during the remodeling of human ductus arteriosus. Apoptosis, however, is not limited to cell elimination during embryonic development. In recent years, apoptosis has been implicated in cardiovascular disease. We want to focus on the detection of apoptosis in the atherosclerotic plaque.

The initial description of apoptosis was based on morphological features. Several useful biochemical and immunohistochemical detection methods were subsequently introduced. Wyllie in 1980 described fragmentation of nuclear DNA into multiples of 180 bp as the result of endonuclease activation. When fragmented DNAs were electrophoresed in an agarose gel, they separated into a characteristic DNA “ladder” pattern. Gavrieli et al described another widely used method, in which DNA breaks in apoptotic nuclei were marked by dUTP-biotin transferred to the free 3′-end of the cleaved DNA. Because terminal deoxynucleotidyl transferase (Tdt) was used to transfer dUTP-biotin by nick end labeling, a more convenient acronym, TUNEL, was coined to describe this procedure.

Recently, the detection of DNA fragmentation by the use of the TUNEL technique or in situ nick translation has become a standard technique for the detection of apoptosis in tissue sections. This technique is particularly interesting in those diseases that are characterized by low values of cell replication and cell death (eg, the formation of atherosclerotic plaques, which are characterized by slow progression).

Although the TUNEL technique is a widespread procedure to detect apoptosis, it is prone to some pitfalls. It was demonstrated that the TUNEL technique could label non-nuclear structures in atherosclerotic plaques. This problem can be avoided by pretreatment of the histological sections with either EDTA or citric acid. The ultrastructural equivalents of these nonnuclear structures are cytoplasmic remnants that can calcify. The vesicles are similar to matrix vesicles that are present in the cartilage epiphysis of long bones.

Another drawback of the TUNEL technique is that nuclei can be labeled nonspecifically due to the proteinase K

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pretreatment or differences in fixation and prefixation times. In accordance with Hegyi et al., we also found that the technique is very sensitive and therefore needs careful titration of proteolytic pretreatment and TdT concentration, otherwise a high fraction of nonapoptotic nuclei will be labeled. In a recent study, a molecular explanation for this phenomenon was found. It was demonstrated in this study that besides apoptotic nuclei, nonapoptotic nuclei that show signs of active gene transcription are labeled by the TUNEL technique. These cells are still active and are transcribing genes that might be related or completely unrelated to the apoptotic cell death pathway. In a true apoptotic cell, the nuclear DNA is cleaved in oligonucleosome-sized fragments, and processes like RNA transcription and splicing are abolished. Moreover, even in the early "execution" phase of apoptosis, caspasases (CPP-32 and ICH-1L) cleave the 70-kDa protein component of splicing factor U1 small nuclear ribonucleoprotein. The loss of RNA splicing can be considered as an early step in the execution phase of apoptosis. Therefore, it is evident that nuclei that are TUNEL-positive and show signs of high RNA synthesis and splicing activity are clearly not in the execution phase of apoptosis. In general, these nuclei also do not show the classic criteria of apoptosis. The fact that the TUNEL technique labels nuclei with high RNA synthetic activity is not surprising, since in the past, several groups have employed a modification of the DNA in situ nick translation method to allow the in situ detection of sites of active gene transcription. Therefore, the TUNEL technique, though useful for detecting the execution phase of apoptosis, should always be combined with additional techniques such as markers of transcription and morphological criteria.

**Apoptosis in Atherosclerotic Plaques: Which Values Can We Trust?**

Different studies have demonstrated that cells can die in atherosclerotic plaques through apoptosis. However, a large variation in the percentage of TUNEL-positive nuclei has been found, ranging from <2% up to 30%. In a recent article, values up to even 60% have been reported. The TUNEL technique labels the execution phase of apoptosis, which in cell culture takes <6 hours. Some of the reported values would indicate that plaques are in an imminent state of collapse, which is certainly not the case, as remarked by Newby and George. This suggests that the TUNEL technique is not without pitfalls as already discussed in the previous paragraph. However, it is now without doubt that cells can die within atherosclerotic plaques through apoptosis.

Another important item is that the level of apoptotic cell death is strongly related to the stage of development of the atherosclerotic plaque. Therefore, large variability can be expected when atherosclerotic plaques of different stages are compared. In general, adaptive intimal thickening and fatty streaks show very little apoptosis, whereas advanced atherosclerotic plaques show foci of apoptosis (the Figure). Most of these foci are associated with regions of macrophage infiltration.

Isner et al and Han et al found evidence for apoptotic cell death in primary atherosclerotic lesions and restenotic lesions. Apoptotic cell death was positively linked to cell replication. Restenotic lesions, showing high replication rates, also demonstrated more apoptotic nuclei. Bennett et al showed in an in vitro study that proliferating smooth muscle cells (SMCs) show more apoptotic cell death than do nonproliferating SMCs. A similar result was found by Bochaton-Piallat et al in the intimal thickening induced after endothelial denudation of the rat aorta. Bauriedel et al, however, found that human restenotic intimal thickenings showed less apoptotic cell death than did primary advanced human atherosclerotic plaques. A major difference could be the presence of replicating foam cells of macrophage origin in advanced human atherosclerotic plaques. In a study of vein graft atherosclerosis, which is considered a form of accelerated human atherosclerosis, a consistent association was found between foam cell accumulation and SMC death in the fibrous cap. These findings confirm the studies of Imai and Thomas of diet-induced lesions in cerebral atherosclerosis in swine that were published some 25 years ago. These authors studied the induced atherosclerotic lesions extensively by transmission electron microscopy and found SMC death in the plaques. These authors described these changes as necrosis of the cells, although their description of the nuclear and cytoplasmic changes fulfilled the criteria of apoptotic cell death.

**Apoptosis in Atherosclerotic Plaques: Which Cell Type?**

The significance of apoptotic cell death of macrophages and SMCs could be very different. The disappearance of macrophages by apoptosis could have a positive effect on plaque stabilization. Indeed, the death of macrophages would lead to decreased breakdown of the collagen fibers. On the contrary, the disappearance of SMCs from the fibrous cap or other vulnerable regions of the plaque could lead to destabilization of the plaque. Therefore, it is essential in studies on apoptosis in atherosclerosis to clearly define which cell type is involved. This discernment, however, is not always possible, since most of the apoptotic cells lose their differentiation markers during the process of apoptosis. Björkerud and...
Björkerud have found that macrophages, as well as lymphocytes and SMCs, can be labeled by the TUNEL technique in atherosclerotic plaques. Geng and Libby, and Han et al have found similar results, which indicate that both macrophages and SMCs can die in atherosclerotic plaques through apoptosis. We have recently confirmed that the TUNEL-positive nuclei and nuclear fragments belong to macrophages and SMCs. However, a significant fraction of the labeled nuclei and nuclear fragments could not be stained by CD68 or α-SMC actin, which could reflect a loss of specific markers during apoptosis. A feature of SMCs in atherosclerotic plaques is that they are surrounded by “cages” of thickened basal lamina (pancakelike SMCs). Basal lamina and basement membranes can be stained with a periodic acid–Schiff (PAS) stain. By combining the TUNEL technique with a PAS stain, we could detect TUNEL-labeled nuclei and nuclear fragments that were enclosed by a cage of PAS-positive material, indicating smooth muscle apoptosis. Moreover, clusters of TUNEL-negative cytoplasmic remnants, which were enclosed by thickened basal laminae, were present. Transmission electron microscopy confirmed the presence of small, membrane-bound vesicles of varying size that were shed from SMCs and the SMCs that had died by disintegration into a myriad of vesicles. These vesicles were enclosed by prominent cages of basal lamina. These vesicles are similar to the granulovesicular degeneration of SMCs present in cerebral atherosclerosis, as described by Stehbens >20 years ago.

Are SMCs “Programmed” to Die Within the Atherosclerotic Plaque?

Another important consideration is that although the SMCs in the atherosclerotic plaques are programmed to die, additional factors are necessary for completing the cell death pathway. This concept is suggested by the interesting finding that SMCs derived from the atherosclerotic plaque but not from the media die when brought into culture. In a recent study by our group, it was demonstrated that SMCs of human fatty streaks differed from the media and adaptive intimal thickening by the presence of BAX, a pro-apoptotic protein of the BCL-2 family. It was not evident which factor heterodimerized with BAX, since BCL-2 could not be detected. Recent data demonstrate that BCL-XI could be involved. BCL-X is present in the media of normal rat arteries. Interestingly, Pollman et al induced apoptosis in the plaques by the use of an antisense probe against the mRNA of BCL-XI.

The presence of BAX in human fatty streaks indicates that both SMCs and macrophages are programmed to die but that an additional factor is necessary to start the execution phase of apoptosis (the Figure). This execution phase, which is detected by the TUNEL technique, was not detectable in the fatty streaks but was frequent (up to 2%) in the advanced atherosclerotic plaques. Interestingly, Mallat et al found a colocalization of caspase 3 (CPP-32) and apoptotic cells in advanced human atherosclerotic plaques. This fits well with the concept that the caspases (especially caspases 2 and 3) are the “executioners” of the apoptotic cell death pathway. The Fas system has also been studied in atherosclerotic plaques. Vascular SMCs express Fas constitutively, but the presence of the Fas ligand in certain regions could lead to apoptotic cell death in vulnerable regions of the plaque. Interestingly, Han et al have found that some SMCs decrease their Fas expression in atherosclerotic plaques, which could lead to a resistance to cell death via the Fas system. This latter scenario is a good example to illustrate that apoptosis is regulated in mammalian cells by multiple factors, either pro-life or pro-death. We suspect that SMCs within the atherosclerotic plaque become programmed to die (commitment phase) and that additional factors (mainly macrophage- and lipid-derived products) are focally present in the plaque that terminate the cell death program (execution phase).

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