

## REVIEW ARTICLE

# Apoptosis in Vascular Disease

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### Introduction

Apoptosis, or programmed cell death, was first described by Kerr *et al.*<sup>1</sup> in 1972 as a specific type of cell death morphologically distinct from necrosis. Apoptotic cells undergo shrinkage with nuclear condensation and the formation of membrane-bound vesicles called apoptotic bodies. These are phagocytosed by adjacent cells and macrophages without inducing an inflammatory response. In contrast, necrotic cells sustain membrane damage leading to the release of pro-inflammatory intracellular contents. Apoptosis although recognised to be fundamentally important in development also occurs in normal physiological circumstances. Apoptotic vascular smooth muscle cells (VSMCs) have been identified during the physiological regression and closure of the human ductus arteriosus before birth.<sup>2</sup> Similarly, apoptotic VSMCs and endothelial cells are present in umbilical veins and arteries following the significant haemodynamic changes that occur during birth.<sup>3</sup> Such apoptosis appears to be triggered by a reduction in blood flow. Further support comes from experimental studies using immature rabbits which demonstrate that reducing flow in the common carotid artery, by ligating the external carotid, results in both VSMC and endothelial cell apoptosis.<sup>4</sup> The normal blood vessel wall is characterised by a low endothelial cell turnover with a variety of mechanisms affording protection against apoptosis.<sup>5</sup> Indeed, recent data indicate that apoptosis may be important in the pathophysiology of vascular disease. A better understanding of this

process may offer novel avenues for therapeutic intervention.

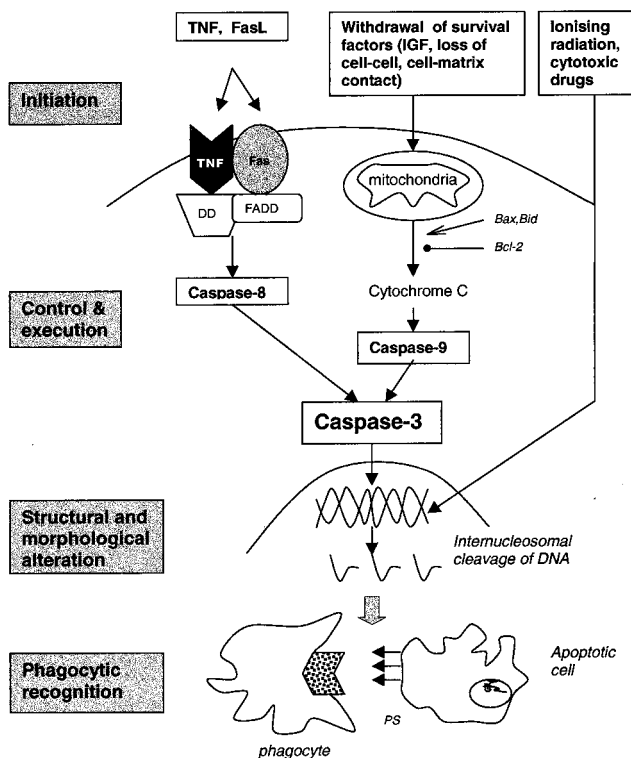
### Molecular Aspects of Apoptosis

Since the original morphological description of apoptosis many molecular mechanisms that characterise the apoptotic pathway have been identified. Apoptosis is controlled by a well-ordered cascade of cellular events that may be divided into four stages (initiation, control/execution, structural/morphological alteration and phagocytic recognition) (Fig. 1). Initiation of apoptosis may occur following the binding of death promoting factors (e.g. Tumour Necrosis Factor (TNF) and Fas Ligand) to cell surface TNF and Fas death receptors or by a withdrawal of survival factors (e.g. reduced insulin-like growth factor, loss of cell–cell and cell–matrix contact) acting via the mitochondria. Alternatively apoptosis may be initiated by ionising radiation or chemotherapeutic agents acting directly on DNA.

The control and execution stage of apoptosis is dependent on the sequential activation of a group of cysteine proteases referred to as caspases. Activation of caspase-8 occurs following binding of the cell surface death-receptors (TNF and Fas).<sup>6</sup> Caspase-9 is activated by cytochrome C released from mitochondria stimulated by the withdrawal of survival factors. Both caspase-8 and 9 can activate the final executioner, caspase-3, which in turn causes the irreversible fragmentation and degradation of DNA.

Control of the execution phase is achieved by anti-apoptotic proteins belonging to the Bcl-2 family (Bcl-2, Bcl-X<sub>L</sub>) inhibiting pro-apoptotic proteins (Bax and

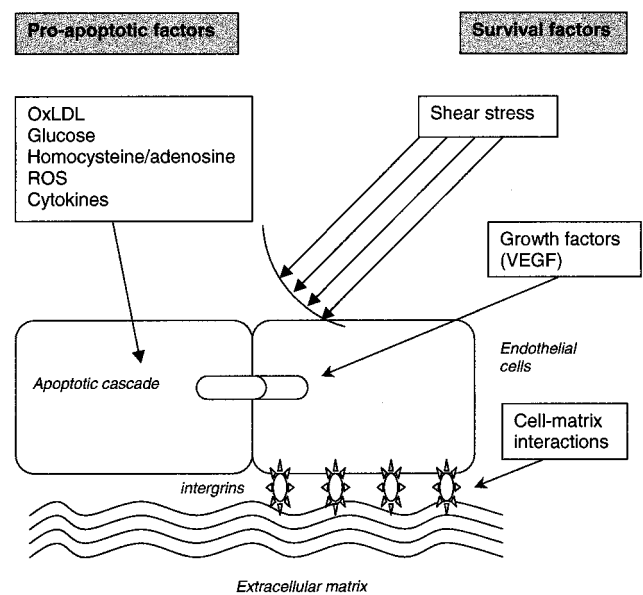
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**Fig 1.** A schematic diagram illustrating four functional stages of apoptosis and the three major pathways through which the cascade of cellular events can proceed. The three pivotal caspases (8, 9 and 3) are shown with caspase-3, the final executioner, causing irreversible DNA damage and loss of cell viability. Control is achieved with the regulatory pro and anti-apoptotic proteins (Bax/Bid & Bcl2 respectively). TNF (Tumour necrosis factor/receptor), Fas L (Fas Ligand), Fas (Fas receptor), DD (TNF death domain protein), FADD (Fas-associated death domain), IGF (Insulin-like growth factor) and PS (Phosphatidylserine).

Bid) that facilitate the release of cytochrome C from the mitochondria. The relative amounts of these proteins determines whether the cell undergoes apoptosis or not. Structural and morphological changes to the cell occur following the internucleosomal fragmentation of DNA leading to an irreversible loss of cell viability. Early apoptotic cells are characterised by externalisation of phosphatidylserine (PS) residues, which are normally situated on the inner leaflet of the plasma membrane. This feature facilitates the specific recognition of apoptotic cells by macrophages enabling their elimination by phagocytosis.

The efficient removal of apoptotic cells can make their identification difficult. Historically the detection and quantification of apoptotic cells has relied on morphological assessment by electron microscopy or light microscopy.<sup>1</sup> Newer techniques rely on the detection of fragmented DNA by terminal transferase mediated dUTP-biotin nick end labelling (TUNEL) or *in situ* nick translation (ISEL).<sup>7</sup> Both methods have become the standard technique for the detection of



**Fig 2.** A schematic summary of pro-apoptotic and survival factors typically affecting the endothelial cell. A loss of survival factors will also precipitate apoptosis. ROS (reactive oxygen species), VEGF (Vascular endothelial cell growth factor).

apoptosis in tissue sections. The major drawback of the TUNEL technique is that nuclei can be stained non-specifically due to differences in cell fixation and also non-nuclear structures in atherosclerotic plaques may be stained. It is recommended that this technique be used in conjunction with an additional morphological method.<sup>5</sup>

Additional methods, suitable for *in vitro* use only, include the detection of externalised phosphatidylserine (PS) residues, that occurs in early apoptosis, with a fluorescein (FITC) conjugate of Annexin-V, a protein that has a high natural affinity for PS. This method allows quantitative assessment of apoptotic cells either by fluorescence microscopy or flow cytometry.

### Pro-apoptotic Factors Implicated in Vascular Cell Apoptosis

*In vitro* studies have shown that extracellular agents, including well characterised cardiovascular risk factors and inflammatory mediators induce apoptosis of endothelial cells and vascular SMCs. (Fig. 2).

#### *Oxidised Low Density Lipoprotein (OxLDL)*

Elevated LDL is a significant risk factor for coronary artery disease. Specifically, its oxidised form (OxLDL)

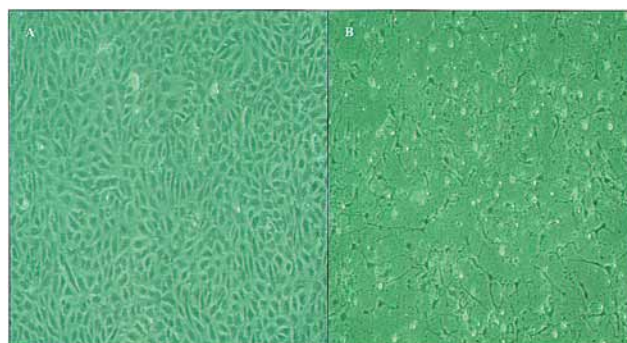
is considered to play a key role in the development of premature atherosclerosis through the formation of foam cells and fatty streaks.<sup>8</sup> OxLDL is known to be cytotoxic to cultured endothelial cells and has also been shown to induce apoptosis, in bovine aortic endothelial cells and human umbilical vein endothelial cells (HUVECs).<sup>9-11</sup> OxLDL-induced apoptosis is inhibited by the addition of the antioxidant vitamins C and E,<sup>12</sup> and is therefore believed to act through the generation of reactive oxygen species. Cultured vascular smooth muscle cells also undergo apoptosis when exposed to oxLDL and oxysterols.<sup>13,14</sup>

#### *Hyperglycaemia/advanced glycation end products*

Endothelial cells cultured with high concentrations of glucose (30 mmol/L) for 48 h show increased apoptosis compared with those incubated in low concentrations of glucose (5 mmol/L).<sup>15</sup> High glucose also appears to induce apoptosis through the generation of hydrogen peroxide and can be inhibited by antioxidants such as ascorbic acid.<sup>16</sup> Similarly high concentrations of advanced glycation end products, the products of non-enzymatic glycation of proteins in the circulation, can induce apoptosis of endothelial cells after 48 h.<sup>17</sup> Interestingly, the lipid-lowering drug pravastatin can prevent hyperglycaemia induced apoptosis<sup>18</sup> which contrasts with the proapoptotic effects of the lipophilic statins (atorvastatin, simvastatin and lovastatin) on vascular smooth muscle cells.<sup>19</sup>

#### *Homocysteine*

Hyperhomocysteinaemia is, an independent risk factor for cardiovascular disease, and, is known to cause endothelial dysfunction.<sup>20</sup> We have recently shown that homocysteine in combination with adenosine, an important substrate in homocysteine metabolism, can induce apoptosis of cultured HUVECs and human long saphenous vein endothelial cells after as little as 4 h incubation<sup>21</sup> (Fig. 3). These effects can be prevented by vitamin B6, B12 and folate,<sup>22</sup> cofactors in homocysteine metabolism and currently used as homocysteine-lowering agents in clinical trials. The latter finding may represent an important mechanism by which vitamin supplementation can reduce vascular injury associated with hyperhomocysteinaemia.



**Fig. 3.** Phase contrast microscopy of cultured human umbilical venous endothelial cells (HUVECs). A: control cells with a characteristic cobblestone morphology, B: After 4 h incubation with homocysteine and adenosine (0.5 mM) showing contracted apoptotic cells.

#### *Cytokines and inflammatory mediators*

The inflammatory cytokines TNF $\alpha$ , IL-1 $\beta$  and IFN- $\gamma$  have all been identified in human atherosclerotic plaques.<sup>23</sup> TNF $\alpha$  causes endothelial cell apoptosis<sup>24</sup> through the activation of caspase-3 and may be inhibited by specific inhibitors of this enzyme.<sup>25</sup> Interestingly, TNF $\alpha$  can also activate a survival pathway that requires protein synthesis.<sup>26</sup> Inhibitors of RNA transcription or protein synthesis can therefore augment TNF $\alpha$ -induced apoptosis of endothelial cells. In contrast TNF $\alpha$ , does not cause apoptosis of cultured human smooth muscle cells on its own, but does so when combined with IL-1 $\beta$  and IFN- $\gamma$ .<sup>27</sup> Lipopolysaccharide also induces human endothelial cell apoptosis *in vitro* that can be prevented by antioxidants.<sup>12</sup> This effect also occurs *in vivo*, but unlike the *in vitro* setting is dependent on TNF $\alpha$  release.<sup>28</sup> Angiotensin II, although promoting growth of VSMCs, induces endothelial apoptosis *in vitro* in a dose dependent manner.<sup>29</sup>

#### *Reactive oxygen species*

Elevated reactive oxygen species (ROS) are an early hallmark of atherogenesis.<sup>30</sup> *In vitro* evidence indicates that ROS, the by-product of aerobic metabolism, induce endothelial and smooth muscle cell apoptosis.<sup>31</sup> ROS may be produced in the vessel wall by macrophages within the atherosclerotic plaque or endogenously by human endothelial and vascular smooth muscle cells. The proatherosclerotic and proapoptotic factors oxLDL, TNF $\alpha$ , glucose and angiotensin II, all induce endogenous ROS whose effects can be prevented by antioxidants.<sup>30</sup>

## Survival Factors Implicated in Preventing Vascular Cell Apoptosis

### *Shear stress and nitric oxide*

Cultured endothelial cells (HUVECs) undergo a basal level of apoptosis in the absence of flow, which can be inhibited by mimicking flow conditions in a perfusion chamber.<sup>32</sup> Laminar flow generates shear stress at the endothelial cell surface that can prevent apoptosis induced by different stimuli including TNF $\alpha$ , oxLDL and ROS. This inhibition is mediated by shear stress-induced release of nitric oxide (NO) that subsequently inactivates caspase-3.<sup>33</sup> In the normal vasculature the shear stress associated with laminar flow causes a continuous production of NO by endothelial cells, providing protection from injury and apoptosis. However atherosclerotic plaque-prone areas are typically sites of turbulent blood flow and low shear stress, and are associated with increased cell turnover,<sup>34</sup> which is most probably secondary to increased apoptosis.<sup>35</sup>

### *Growth factors*

Cultured endothelial cells deprived of growth factor undergo apoptosis.<sup>36</sup> Addition of vascular endothelial growth factor (VEGF) can inhibit apoptosis induced by TNF $\alpha$ ,<sup>37</sup> ionising radiation<sup>38</sup> and disruption of the extracellular matrix.<sup>39</sup> Angiopoietin-1 can also prevent apoptosis in growth factor deprived endothelial cells.<sup>40</sup> Basic fibroblast growth factor (FGF-2) prevents lipopolysaccharide induced endothelial cell apoptosis *in vivo* and serum and growth factor deprivation induced apoptosis *in vitro*.<sup>28,36</sup> In cultured human vascular smooth muscle cells several growth factors (insulin-like growth factor, platelet derived growth factor, basic fibroblast growth factor, and transforming growth factor) inhibit apoptosis especially under low serum conditions.<sup>41-44</sup>

### *Atheroprotective factors*

Oestrogen is an established atheroprotective hormone, which is known to produce beneficial changes in lipid profile and the regulation of vascular tone. Oestradiol also maintains endothelial integrity by inhibiting endothelial apoptosis induced by TNF $\alpha$ .<sup>45</sup> Antioxidant vitamins C and E, especially in combination, prevent apoptosis induced by oxLDL, TNF $\alpha$ , and glucose.<sup>30</sup>

## *Cell-matrix interactions*

The survival of endothelial cells and vascular smooth muscle cells is dependent on contact with adjacent cells and the extracellular matrix (Fig. 2). This is mediated by cellular adhesion molecules such as the integrins, which also function as signalling molecules. Apoptosis occurs when these contacts are lost.<sup>46</sup> Under normal circumstances the extracellular matrix generates survival signals that either suppress apoptotic pathways or lead to an increase in the activity of anti-apoptotic pathways such as the Bcl-2 family of proteins.<sup>47</sup>

## Apoptosis and Vascular Disease

### *Plaques*

Early studies in cholesterol-fed swine have shown cell death to be a major component of atherosclerotic plaque development.<sup>48</sup> *In situ* techniques (TUNEL) have since confirmed the presence of apoptotic smooth muscle cells, T-lymphocytes, and macrophages in human atherosclerotic plaques.<sup>27,49,50</sup> More recently Tricot *et al.*<sup>51</sup> have examined carotid endarterectomy specimens and shown that apoptotic luminal endothelial cells occur with a greater prevalence in the post-stenotic area, a region of low flow and low shear stress.

Apoptotic cell death within plaques, as determined by the TUNEL technique, ranges from <2% to 30% and is related to the stage of the atherosclerotic plaque.<sup>27,49,50,52-55</sup> Very little apoptosis occurs in intimal thickening and fatty streaks with the majority of apoptotic cells occurring in advanced atherosclerotic plaques within regions of macrophage infiltration.<sup>5</sup>

Although the above evidence indicates that apoptosis does occur within human atherosclerotic plaques, other authors have used electron microscopy to show that despite >10% TUNEL-positive nuclei, the vast majority of injured and disintegrating cells within plaques display typical features of cells undergoing cell death by necrosis.<sup>56-58</sup>

Although the relative importance of cell death by apoptosis versus necrosis is unknown we can speculate that apoptosis may allow plaque stabilisation. Apoptotic removal of T-cells and macrophages, which commonly infiltrate the shoulder region of the fibrous cap, would reduce matrix metalloproteinase (MMP) synthesis and extracellular matrix breakdown without an accompanying inflammatory reaction. Conversely death of vascular smooth muscle cells in plaques,

either through apoptosis or necrosis, may lead to a weakening of the fibrous cap as a consequence of reduced collagen and extracellular matrix synthesis following the loss of smooth muscle cells.

Apoptosis of luminal endothelial cells<sup>51</sup> in atherosclerotic plaques may initiate plaque erosion with the subsequent promotion of platelet aggregation and thrombosis. Apoptotic vascular cells themselves may also lead to increased plaque thrombogenicity. Both apoptotic smooth muscle cells and endothelial cells expose phosphatidylserine residues on their surface in early apoptosis, which in the presence of factor V and VII, can act as a substrate for the generation of thrombin.<sup>59</sup> Additionally apoptotic monocytic cells have enhanced tissue factor activity.<sup>60</sup> Mallat *et al.*<sup>61</sup> have also identified increased tissue factor expression around apoptotic cells within the central necrotic core of plaques, indicating that tissue factor is shed from apoptotic cells via apoptotic microparticles.

#### *Restenosis*

Apoptosis of smooth muscle cells will occur following balloon injury in animal models. An initial apoptotic response after 30 min<sup>62</sup> is followed by persistent apoptosis after two weeks, the latter associated with the regulation of intimal thickening.<sup>63</sup> Studies of human restenotic plaques indicate there may be an increased apoptotic rate, but there is no consensus. Isner *et al.*<sup>52</sup> found an increased apoptotic rate in human restenotic plaques, whereas Bauriedel *et al.*<sup>64</sup> noted reduced levels of apoptotic cells in restenotic plaques compared with primary plaques.

#### *Aneurysms*

Reduced smooth muscle cell density within the elastic media of human aneurysmal wall is associated with increased smooth muscle cell apoptosis.<sup>65-67</sup> Further evidence has shown that infiltrating T cells express cytotoxic mediators such as cytokines, perforin and Fas/Fas ligand, which are capable of inducing apoptosis. It is hypothesised that these might contribute to the elimination of smooth muscle cells, a source of collagen and elastin, thereby impairing repair and maintenance of the arterial extracellular matrix tending to favour aneurysmal wall expansion.

#### *Transplant arteriopathy*

Medial SMC loss prior to the development of occlusive and intimal proliferative lesions is characteristically found in transplant arteriopathy. Experimental evidence using rat arterial allografts suggest that this SMC loss is due to apoptosis.<sup>68</sup> Endothelial damage, also seen in transplant arteriopathy, appears to be due to a Fas-based apoptotic pathway.<sup>69</sup>

### **Therapeutic Potential**

Apoptotic cells are quickly eliminated by neighbouring phagocytic cells making *in vivo* identification difficult. Our current understanding of apoptosis in vascular disease therefore depends almost entirely upon *in vitro* and *in situ* data. However, recent techniques, which capture shed microparticles released by apoptotic cells, have been used to show increased apoptosis in acute coronary artery syndromes.<sup>70</sup>

#### *Induction of apoptosis*

Atherosclerotic plaques in cholesterol-fed rabbits regress when neointimal cell apoptosis is induced by inhibiting the antiapoptotic protein Bcl-x.<sup>71</sup> Plaque regression also occurs following administration of L-arginine to induce macrophage apoptosis via NO release.<sup>72</sup> Interestingly, Schaub *et al.*<sup>73</sup> found that when vascular smooth muscle cell apoptosis is induced, through the overexpression of death domain proteins, macrophages are recruited and neointimal progression rather than regression occurs.

Various attempts to induce apoptosis have been tried in animal models of neointima formation and restenosis following angioplasty. Transfection of suicide genes into smooth muscle cells or direct delivery of pro-apoptotic ligands (Fas-ligand) into the vessel wall induce smooth muscle cell apoptosis and a significant reduction in neointima formation in rabbit arteries following angioplasty.<sup>74</sup> Adopting this approach in the human clinical setting may however, risk the development of aneurysms.

#### *Inhibition of apoptosis*

Currently there is no information on the effect of inhibiting apoptosis on the progression or complications of vascular disease. *In vitro* and *in situ* data

indicate that apoptosis of many cell types occurs in human atherosclerotic plaques, and aneurysmal wall. Strategies to inhibit apoptosis in these cell types may be the best approach to limit plaque erosion, thrombosis, progression and aneurysmal development. Potential therapeutic options include the use of anti-apoptotic agents known to inhibit apoptosis *in vitro* including NO, growth factors (VEGF), angiotensin converting enzyme inhibitors, antioxidants and vitamins (B<sub>6</sub>, B<sub>12</sub> and folate) as well as controlling the levels of established cardiovascular risk factors (lipids, glucose, and homocysteine).

### Conclusion

Despite the wealth of *in situ* and *in vitro* data demonstrating that apoptosis not only occurs in vascular disease, but can be induced by established cardiovascular risk factors, there is little evidence in humans to indicate whether apoptosis is beneficial or harmful. Direct modulation of apoptosis may provide therapeutic avenues to alter the pathophysiology of vascular disease. However, to date, despite successes in some animal models, their applicability to human vascular disease remains unknown.

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